
**UNIVERSIDAD AUTÓNOMA DE MADRID**

**DEPARTAMENTO DE BIOLOGÍA MOLECULAR**

# *The miR-17-92 cluster counteracts quiescence and chemoresistance of pancreatic cancer stem cells*

**Michele Cioffi**

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# *The miR-17-92 cluster counteracts quiescence and chemoresistance of pancreatic cancer stem cells*

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*Dedicado a Serena,*

*a mi familia y*

*a mis amigos incondicionales*

# Acknowledgements

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# SUMMARY

Pancreatic ductal adenocarcinoma is the fourth most frequent cause for cancer-related deaths due to late diagnosis and limited response to chemotherapy. As one of the identified drug resistance mechanism, a distinct subpopulation of cancer stem cells (CSCs) has not only been shown to promote tumor progression and metastasis, but has also been implicated in tumor relapse based on intrinsic drug-resistance. Therefore, advancing our understanding on the mechanism of drug resistance in CSCs versus non-CSCs could lead to more effective therapeutic strategies. Since CSCs and non-CSCs share an identical genetic background, we studied the epigenetic regulation of CSCs as the defining regulatory machinery of CSCs. Using high-throughput miRNA expression analysis; we identified a panel of common miRNAs to be consistently underrepresented in Gemcitabine-treated tumors compared to untreated tumors. Subsequent gain-of-function and loss-of-function experiments demonstrated that the miR-17-92 cluster encoding six related microRNAs as the most strikingly suppressed miRNA family in chemoresistant CSCs is of crucial relevance for CSC function. Specifically, lentivirus-mediated over-expression of the miR cluster in CSCs significantly reduced their self-renewal, *in vivo* tumorigenicity, and resistance to Gemcitabine by down-regulation of multiple key genes belonging to the Nodal/TGF-β1 signaling cascade such as ALK4, TGFBR2, SMAD2, SMAD4 as well as direct inhibition of Nodal/TGF-β1-responsive genes p21, p57 and TBX3. MiR-overexpression in CSCs also resulted in their enhanced proliferation, which eventually resulted in CSC exhaustion during serial transplantation via down-regulation of p21 and p57. Thus, our study identifies the miR-17-92 cluster as an important family of miRNAs that play a crucial role in CSC biology including chemoresistance. Our findings indicate potential for developing modulators of this cluster to overcome drug resistance in pancreatic CSCs and eventually improve the miserable outcome of patients with pancreatic ductal adenocarcinoma.

# RESUMEN

El adenocarcinoma pancreático es la cuarta causa principal de muerte relacionada con el cáncer debido a su diagnostico tardío y su mala respuesta a la quimioterapia. Uno de los posibles mecanismos de quimioresistencia que ha sido descrito se basa en la existencia de un subconjunto de células tumorales con propiedades troncales, denominadas células troncales de cáncer (CSC), las cuales impulsan el crecimiento del tumor de páncreas, metástasis y quimioresistencia. Por lo tanto, si podemos progresar en el conocimiento del mecanismo de resistencia a la quimioterapa en las células troncales de cáncer, probablemente nos ayudaría a desarrollar terapias más eficientes. Debido a que las células troncales de cáncer y las células cancerígenas comparten un perfil genético idéntico, hemos estudiado la regulación epigenética de las CSCs para definir el mecanismo de regulación. Utilizando el análisis de expresión de los genes de miRNA, hemos identificado un grupo de miRNAs comunes que de manera consistente se encontraban poco expresados en aquellos tumores que habían sido tratados con Gemcitabina comparándolo con los tumores sin tratar. Los experimentos posteriores de ganancia y perdida de funcionalidad, demostraron que el miR-17-92 cluster codificaba seis microRNAs que se encontraban suprimidos en las CSCs quimioresistentes, lo cual era de crucial relevancia en la funcionalidad de las CSCs.

Por consiguiente, al sobrexpresar el miR cluster mediante el uso de un lentivirus en las CSCs, observamos que se reducía de manera significativa la capacidad de auto-replicación, tumorigenicidad in vivo y se reducía la resistencia a la Gemcitabina via inhibición de la expresión de genes clave que pertenecen a la vía de señalizaciónn de Nodal/ TGF-β1 como ALK4, TGFBR2, SMAD2, SMAD4, así como también otros genes que responden a la inhibición tales como p21, p57 y TBX3. La sobreexpresión de miR en las CSCs también resultó en un aumento de la proliferación, lo cual eventualmente resultó en una disminución de la capacidad de auto-replicació en pases seriados debido a la reducción de la expresión de los genes p21 y p57. En resumen, nuestro estudio identifica el miR-17-92 cluster como una familia de miRNAs muy importante que juega un rol crucial en la biología de las CSC incluida la quimioresistencia. Nuestros hallazgos muestran un fuerte potencial para el posible desarrollo de modulaodores de este cluster para poder suprimir la resistencia de las CSCs, y así finalmente, poder mejorar el pronóstico tan miserable de los pacientes con adenocarcinoma pancreático.

# ABBREVIATIONS

| | |
|---|---|
| **ABCG2** | ATP-binding cassette sub-family G member 2 |
| **ALDH-1** | Aldehyde dehydrogenases family 1 |
| **ALK-4** | Activin Receptor-Like Kinase 4 |
| **BSA** | Bovine Serum Albumin |
| **bFGF** | Basic fibroblast growth factor |
| **CK19** | Cytokeratin 19 |
| **hCNT** | (human) Concentrative nucleoside transporter |
| **CSC** | Cancer Stem Cell |
| **CSCs** | Cancer Stem Cells |
| **CXCR4** | Chemokine receptor type 4 |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DMEM/F12** | Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 |
| **DNA** | Deoxyribonucleic acid |
| **DNP** | 2,4-Dinitrophenol |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **hENT** | (human) Equilibrative nucleoside transporter |
| **EpCAM** | Epithelial cell adhesion molecule |
| **5-FU** | 5-Fluoracil |
| **FACS** | Fluorescent-activated cell sorting |
| **FBS** | Fetal bovine serum |
| **FFPE** | Formalin-Fixed, Paraffin-Embedded |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GEM** | Gemcitabine |
| **HPF** | High power fields |
| **HRP** | Horseradish peroxidase |
| **KRAS** | V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| **mRNA** | messenger ribonucleic acid |
| **HNU mice** | Athymic Nude-Foxn1nu |
| **NSG mice** | NOD scid gamma mice |
| **PDAC** | Pancreatic ductal adenocarcinoma |
| **PaCSCs** | Pancretic cancer stem cells |
| **PBS** | Phosphate buffered saline |
| **PEN** | Penicillin |
| **PVDF** | Polyvinylidene difluoride |
| **RT-qPCR** | quantitive real time polymerase chain reaction |
| **RIPA** | Radioimmunoprecipitation assay |
| **RNA** | Ribonucleic acid |
| **RPMI** | Roswell Park Memorial Institute medium |
| **SSEA-1** | Stage-specific embryonic antigen-1 |
| **STREP** | Streptomycin |
| **SOC** | Standard of care |
| **SDS-PAGE** | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **SDF-1** | Stromal cell-derived factor 1 |
| **TBS** | Tris-buffered saline |
| **TGF-ß1** | Transforming growth factor beta 1 |

# INTRODUCTION

## 1. THE PANCREAS

The pancreas is a gland organ located transversely across the posterior wall of the abdomen, at the back of the epigastric and left hypochondriac regions. In adult humans it length varies from 15 to 25 cm., and its weight from 70 to 150 gr. The name pancreas derived from Greek roots "pan" meaning "all" and "creas" meaning "flesh or meat", and is based on the fact that this organ lacks bones and cartilage (Slack, 1995, Buchler, 2002). The entire organ can be divided into four different regions: head, neck, body and tail **(Figure I1)**. The head of the pancreas is the largest part and lays on the right side of the abdomen where the stomach is attached to the first part of the small intestine (the duodenum). The neck is a slight constriction between the head and the body that extends to the left side of the abdomen next to the spleen while its left extremity gradually tapers to form the tail. There is a duct that runs the length of the pancreas, and several small branches from the glandular tissue join it. The end of this duct is connected to a similar duct that comes from the liver, which delivers bile to the duodenum.

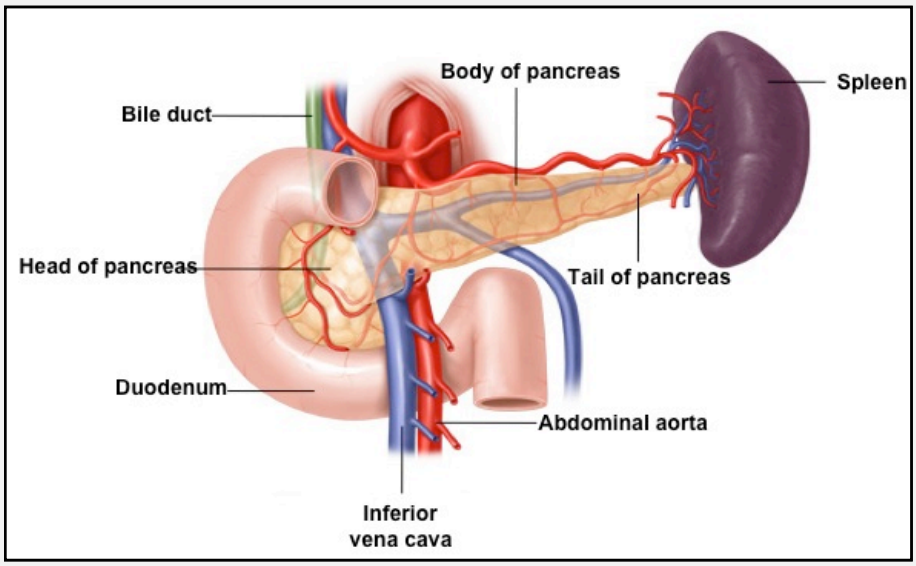



**Figure I1**. Representative illustration showing the different regions of the pancreas: head, neck body and tail. *Adapted from Lippincott Williams and Wilkens Atlas of Anatomy, 2009.*

The pancreas is classified as a heterocrine gland because it contains both endocrine and exocrine glandular tissue. The exocrine tissue represent about 85% of the pancreas by weight

and secrete digestive enzymes, water and $NaHCO_3$ into the intestine, while endocrine tissue makes up the other 15% and secretes hormones into the blood stream, such as insulin (Pandol, 2010).

## 1.1. The endocrine pancreas

The endocrine pancreas is composed by cells within the pancreas that synthesize and secrete hormones. The endocrine portion of the pancreas takes the form of many small clusters of cells called islets of Langerhans or, more simply, islets. In standard histological sections of the pancreas, islets are seen as relatively pale-staining groups of cells embedded in a darker-staining exocrine tissue (Frohman, 1969). Pancreatic islets house three major cell types, each of which produces a different endocrine product:

- Alpha cells (A cells) secrete the hormone glucagon that elevates the amount of glucose in the blood by promoting gluconeogenesis and glycogenolysis in the liver binding glucagon receptor (Youngs, 1972)
- Beta cells (B cells) are the most abundant of the islet cells and produce insulin that is necessary to remove excess glucose from the blood (Krahl, 1974).
- Delta cells (D cells) secrete the hormone somatostatin which is also produced by a number of other endocrine cells in the body and via interaction with G protein-coupled somatostatin receptor, inhibits the release of numerous secondary hormones in the gastrointestinal system and pituitary gland (Gahete et al.)
- PP cells which secrete pancreatic polypeptide (PP) (Slack, 1995)
- Epsilon cells represents <1% and produces gherlin, a potent stimulator of growth hormone secretion from the pituitary gland (Kojima et al., 1999).

Interestingly, the distribution of the several cell type is very specific: beta cells occupy the central portion of the islet and are surrounded by a "rind" of alpha and delta cells. Moreover, the islets are richly vascularized, allowing the rapid secretion of the hormones to the circulation **(Figure I2)**. Although islets comprise only 1-2% of the mass of the pancreas, they receive about 10 to 15% of the pancreatic blood flow. Additionally, they are innervated by

parasympathetic and sympathetic neurons, and nervous signals clearly modulate secretion of insulin and glucagon. (Pandol, 2010).

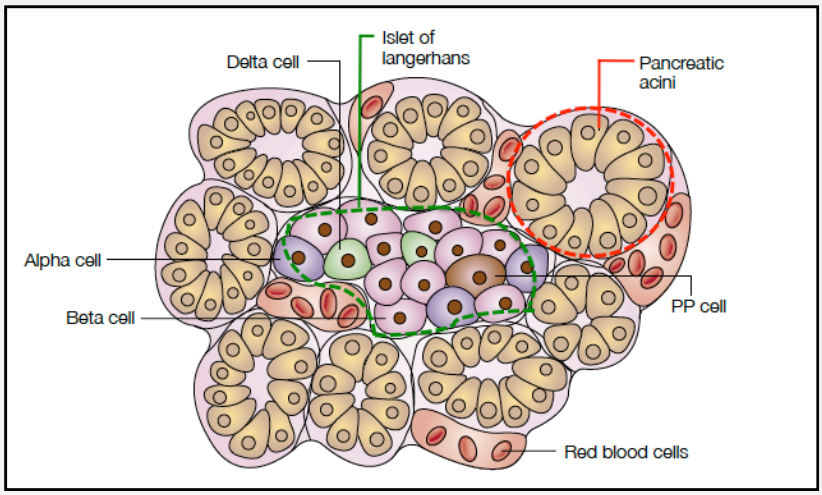



**Figure I2**. Representative illustration of an islet of Langerhans within the exocrine mass representing different types of endocrine cells. *Adapted from (Bardeesy and DePinho, 2002)*

The most important disease of endocrine pancreas disease is the Diabetes mellitus characterized by high blood sugar levels due to disruption of insulin producing beta cells (Type I – insulin dependent) or due to a variety of different events where different organs no longer respond to the effects of insulin (Type II or non insulin dependent) (Slack, 1995).
In addition to diabetes, the endocrine pancreas can also suffer transformation of neuroendocrine cells, resulting in pancreatic neuroendocrine tumours that can be either functional (produce hormones) or non-functional (produce no hormones). Most functional neuroendocrine tumours are benign. However, 90% of non-functional neuroendocrine tumours are cancerous. The frequency of these tumours is very low (1-2%) while 95% are adenocarcinomas, which arises from the exocrine pancreas (Kloppel and Heitz, 1988).

## 1.2. The exocrine pancreas

The exocrine part of the pancreas is composed by tubuloacinar glands organised like bunches of grapes and surround the islets of Langerhans; the main ducts, blood vessels and nerve fibers

are surrounded by connective tissue. It is mainly composed of two different types of cells: acinar and ductal cells. Acinar cells are pyramidal epithelial cells that secrete the digestive enzymes of the pancreatic juice, such as proteases, amylases, lipases and nucleases into the acinus lumen, which are essential for transforming and processing food into nutrients that can be absorbed by the intestine. Ductal cells are cuboidal to pyramidal in shape and secrete bicarbonate and mucus into the enzyme mixture. This enzyme mixture is secreted into the ducts, which empty into the duodenum **(Figure I3)** (Pandol, 2010, Bardeesy and DePinho, 2002, Slack, 1995).

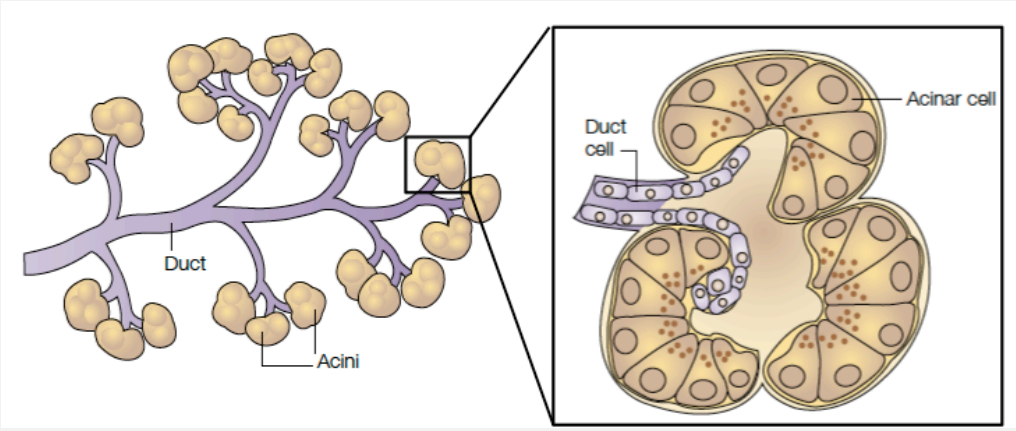



**Figure I3**. Representative illustration of the exocrine pancreas representing the different cell types (i.e. duct and acinar cells) in the acinus. *Adapted from (Bardeesy and DePinho, 2002)*

Apart from acinar and ductal cells, there are several resident cells in the exocrine pancreas including pancreatic stellate cells (PaSCs). They are present in the periacinar space and have long cytoplasmic processes that encircle the base of the acinus, but also in perivascular and periductal regions of the pancreas participate in chronic pancreatitis and pancreatic cancer. In these disorders, PaSCs participate in disease pathogenesis after transforming from a quiescent state into an "activated" state. In normal conditions are involved in tissue repair activities; however, under pathologic conditions, they are "activated" into a high proliferative myofibroblast-like cell that proliferates and secretes extracellular matrix proteins and growth factors, which can remodel the microenvironment (Omary et al., 2007).

Pancreatitis is an inflammation of the pancreas where digestive enzymes are activated before they are secreted into the duodenum and begin attacking the pancreas. Acute pancreatitis is is a sudden attack causing inflammation and necrosis of the pancreas parenchyma that in general is a transient and mild disease. In contrast, chronic pancreatitis is persistent and uncontrolled inflammation that leads to permanent deterioration of the structure and function of the pancreas with extensive fibrosis. The most common cause is long-term alcohol abuse, but also smoking, genetic and autoimmunity (Buchler, 2002).

The other major disease of the exocrine pancreas is the Pancreatic Ductal Adenocarcinoma (PDAC), which is believed to arise from the exocrine pancreas and is the most frequent type of pancreatic cancer (95% of cases).

## 2. PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC) is the deadliest solid cancer and currently the fourth most frequent cause of cancer-related deaths world-wide (Jemal et al., 2010). In contrast to the general trend of decreasing incidences for most cancers, the incidence and death rates for PDAC continue to increase ("Cancer Facts & Figs. 2011", American Cancer Society, www.cancer.org). Similar trends are noted for Europe, where cancer-related deaths have declined for most other cancers, but have increased by 20% for PDAC (WHO European Health Report 2012, (Han and Von Hoff, 2013).

Has been estimated that 10% of PDAC cases are associated with an inherited predisposition based on familial clustering (Schenk et al., 2001); around 20% of PDAC-prone familial cases shows several germline mutations, including those targeting the tumor suppressor genes *INK4A, BRCA2,* and *LKB1,* the DNA mismatch repair gene *MLH1* (Jaffee et al., 2002). Due to the low penetrance of PDAC and the typical age of onset associated with the above germline mutations, these genetic lesions appear to impact malignant progression of precursor lesions rather than cancer initiation. Supporting this hypothesis, *INK4A* and *BRCA2* mutations are not detected in the earliest sporadic PDAC premalignant lesions but are only found in the later intermediate or advanced pancreatic intraepithelial neoplasm (PanIN) lesions (Wilentz et al., 1998); (Goggins et al., 2000). Moreover, several genetic syndromes have been associated with an increased risk of pancreatic cancer including familial atypical

multiple mole melanoma (*p16/CDKN2A*) (Lynch et al., 2002), hereditary non-polyposis colorectal syndrome (HNPCC) (Canto et al.) and familial pancreatitis (Lowenfels et al., 1997).

How these genetic conditions lead to PDAC remains not fully understood, but few known risk factors have been directly linked to development of PDAC. Long-standing chronic pancreatitis could promote tumourigenesis in part by promoting the local release of cytokines and reactive oxygen species (ROS) that induce cell proliferation, disrupt cell differentiation states, and select for oncogenic mutations (Hezel et al., 2006).

Moreover, smoking has been identified as clear risk factors since has been demonstrated that smokers have around a 3 times higher risk of developing pancreatic cancer than no-smokers (Hassan et al., 2007). In addiction, diabetes and obesity appear to confer increased risk of PDAC (Everhart and Wright, 1995, Gapstur et al., 2000, Michaud et al., 2001, Berrington de Gonzalez et al., 2003),

PDAC commonly arises in the head of the pancreas with infiltration into surrounding tissues including lymph node, spleen, and peritoneal cavity, preferentially metastasizing to the liver and lungs; even the smallest primary lesions commonly exhibit lympho-vascular invasion, suggesting a propensity for early distant spread. The disease is characterized by extensive desmoplasia with dense stroma due to a severe inflammation and activation of pancreatic stellate cells. The precursor lesions in PDAC can be divided into three groups based on the clinical and histopathologic analysis: mucinous cystic neoplasm that harbor an ovarian-type stroma (MCN), intraductal papillary mucinous neoplasm that grow into larger cystic structures (IPMN) and the most studied pancreatic intraepithelial neoplasia (PanIN) (Maitra et al., 2005). PanINs lesions can be identified and distinguishes based on morphological alterations relative to normal ducts that correlates with an increase in dysplastic growth (Hruban et al., 2001). Starting from normal ductal cells, the cells start to acquire a mucinous and columnar epithelium phenotypes that reflect PanIN-1A which then progress on to PanIN-1B lesions, and on to medium- and high-grade lesions: PanIN-2 and PanIN-3 lesions characterized by nuclear atypia and extensive architectural disorganization and are believed to be the final precursor to PDAC (Mihaljevic et al., 2010). The high-grade PanINs ultimately transform into PDAC with evidence of areas of invasion beyond the basement membrane. During PanIN-to-PDAC progression occurs several gene alterations: 1) telomere shortening that cause genetic instability (Mihaljevic et al., 2010) 2) KRAS mutations are the driving

activating mutations acquired during early events (Avila et al., 2012) and occur in greater than 90% of PDAC patients 3) CDNK2A inactivation brings the P16 loss, which is a regulator of cell cycle during G1-S transition 4) P53 loss is not as frequent as the other mutations, around 50-70%, but its loss represents a deregulation of the cell cycle due to the lack of checkpoints for DNA damage control or apoptosis 5) SMAD4 mutations, present in ~50% of cases, acts also as a tumour suppressor, and its loss or mutated form bring the deregulation of the TGF-β signalling (Hidalgo and Von Hoff, 2012, Morris et al.) **(Figure I4).**

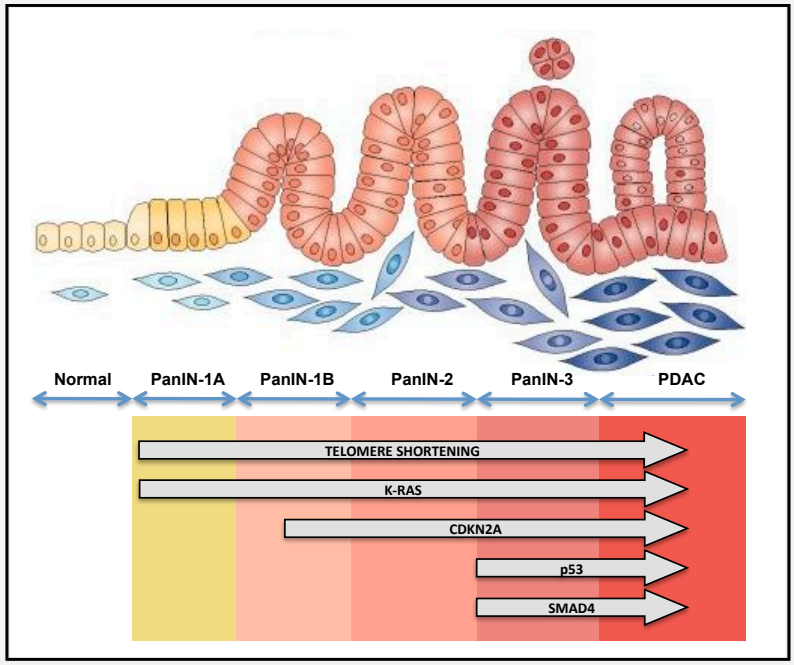



**Figure I4**. Representative illustration of the histopathological changes during PDAC progression (upper panel). Genetic alterations during progression **(lower panel).** *Adapted from (Morris et al.)*

Pancreatic cancer is more common in elderly people than younger people, and only 20% of patients present with potentially curable tumours, largely due to late diagnosis as a consequence of the lack of early symptoms and poor or ineffective diagnostics. Thus, the 1- and 5-year relative survival rates for PDAC are currently 25% and 6%, respectively also because the majority of PDAC patients show extensive metastases in secondary organs including the liver, lungs and bone marrow at the time of diagnosis. Moreover PDAC shows high resistance to both chemo- and radio-therapy that further limits the potential to

effectively treat these patients. Different types of treatment are available for patients with pancreatic cancer. Some treatments are standard (the currently used treatment), and some are being tested in clinical trials. Depending on the stage and location of the cancer as well as on your age, overall health and personal preferences, there are several treatments approaches. The first approach is surgical, aimed to eliminate the tumours using the Whipple procedure, very complex and invasive, in which the head of the pancreas, the gallbladder, part of the stomach, part of the small intestine, and the bile duct are removed. Enough of the pancreas is left to produce digestive juices and insulin. Although the procedure results in a five-year survival of 20%, only ~20% of patients with localized disease can benefit (Philip et al., 2009). If the cancer has spread and cannot be removed, biliary and gastic bypass may be done as palliative surgery to relieve symptoms and improve quality of life.

Chemotherapy may be used at any stage of pancreatic cancer. It is commonly used when the cancer is advanced and can't be removed completely with surgery, however may also be used after the cancer has been removed with surgery to try to kill any cancer cells that have been left behind. This type of treatment is called *adjuvant* treatment that lowers the chance that the cancer will come back later, while *neoadjuvant* treatment is given before surgery to try to shrink the tumour. Advances in PDAC-specific therapies are summarized in **Table I1** and detailed below.

*Gemcitabine:* The introduction of the nucleoside-analogue Gemcitabine, in 1997, improved clinical response with respect to 5-Fluorouracil by increasing overall survival and reducing pain and suffering (Burris et al., 1997). Gemcitabine was subsequently adopted as the standard of care (SOC) and is still the primary chemotherapeutic choice in the clinic. Nevertheless, with a 5-year survival rate of 1–4% and a median survival period of 4-6 months (Ahlgren, 1996, Jemal et al., 2004, Philip et al., 2009, Rosenberg, 1997, Rothenberg et al., 1996, Warshaw and Fernandez-del Castillo, 1992) the prognosis of patients with pancreatic cancer has remained poor.

*Erlotinib:* It is a reversible tyrosine kinase inhibitor, which acts on the epidermal growth factor receptor (EGFR), which is highly expressed and occasionally mutated in various forms of cancer, including pancreatic cancer, which results in uncontrolled cell division. Unfortunately, erlotinib in combination with Gemcitabine has not resulted in a markedly improved median survival (Moore et al., 2007) compared to SOC.

*FOLFIRINOX*: The use of FOLFIRINOX (combination therapy of oxaliplatin, Irinotecan,

fluorouracil, and leucovorin) in patients with metastatic pancreatic cancer has improved overall survival to 11.1 months as compared with Gemcitabine treatment alone (6.8 months). Compared to Gemcitabine, FOLFIRINOX was associated with a survival advantage but had increased toxicity; therefore, only a subpopulation of patients with good performance are likely to benefit from this combination therapy (Conroy et al., 2011).

*Abraxane*: Abraxane is an albumin-bound paclitaxel particles that targets SPARC, which has been associated with poor prognosis, promoting metastasis and angiogenesis. A recent phase III clinical trial combining Gemcitabine with Abraxane (paclitaxel albumin-bound particles), showed prolonged survival with the combination of both treatments with 8.5 (Abraxane and SOC) vs 6.7 (SOC) (Han and Von Hoff, 2013).

Although tremendous efforts have been invested in improving our therapeutic approaches for treating patients with pancreatic cancer, all patients inevitably succumb to the disease. Therefore, new approaches for targeting pancreatic cancer are still desperately needed to pave the way for the development of disease-free treatment regimens (Hermann et al., 2009, Neesse et al., 2010).

**CLINICAL REGIMENS PROVEN TO INCREASE SURVIVAL FOR PATIENTS WITH ADVANCED METASTATIC PANCREATIC CANER**

| Regimen | Control | Median Survival (months) Regimen | Median Survival (months) Control | Reference |
|---|---|---|---|---|
| Gemcitabine | 5-FU | 5.6 | 4.4 | Burris, et al., 1997 |
| Gemcitabine + Erlotinib | *GEM | 6.24 | 5.91 | Moore, et al., 2007 |
| FOLFIRINOX** | *GEM | 11.1 | 6.8 | Conroy, et al., 2011 |
| nab-paclitaxel + gemctibaine | *GEM | 8.5 | 6.7 | Von Hoff, et al., 2012 |

*GEM: gemcitabine **FOLFIRINOX: Folinic acid + 5 FU + Irinotecan + Oxaliplatin

**Table I1**. Current treatments for PDAC cancer. *Adapted from (Han and Von Hoff, 2013)*

## 3. ROLE OF CANCER STEM CELLS

Tumours are generally assessed clinically by histology and by expression of specific markers and in combination with gene expression analysis; this has led to the definition of distinct tumour subtypes. In addition to different tumour subtypes, cells within the tumour population frequently exhibit functional diversity termed tumour heterogeneity (Heppner and Miller, 1983), with some cells exhibiting differences in proliferation and differentiation capacity. The fundamental cellular mechanisms underlying this tumour heterogeneity are subject of intense research activities. Based on the clonal model, a population of mutant cells within the tumour possess a growth advantage and are selected for expand during tumourigenesis, resulting in a homogenous tumour mass (Nowell, 1976), on the other hand the cancer stem cell (CSC) model, postulates a hierarchical organization of cells within the tumour such that only a small subset of "stem-like" cells is responsible for sustaining tumourigenesis and establishing the cellular heterogeneity inherent in the primary tumour (Clarke et al., 2006) **(Figure I5)**. It is important to note that the two models are not mutually exclusive, as CSCs themselves undergo clonal evolution, as shown for leukaemia stem cells

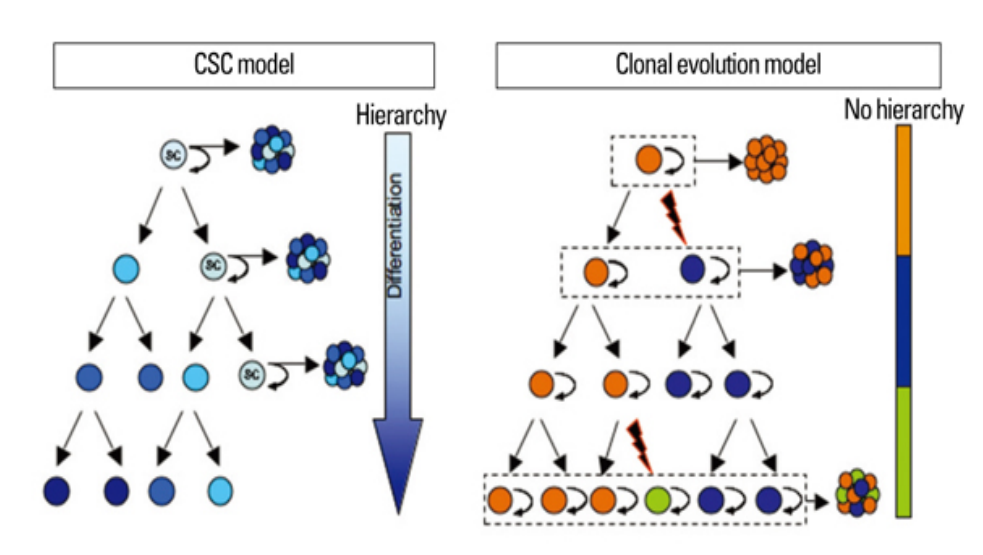



**Figure I5**. Sources of heterogeneity. Cancer Stem Cell Model: Tumours contain different subpopulations of tumourigenic (cancer stem cells) and non-tumourigenic cells organized in a hierarchy. The Clonal evolution model: genetic/epigenetic changes in cancer cells with growth advantages are selected to repopulate the tumour mass. *Adapted from* (Laks et al.).

(Barabe et al., 2007).

In 1997, pioneering studies from John Dick's laboratory identified for the first time leukemia-initiating stem cells (Bonnet and Dick, 1997) followed by landmark studies in breast cancer (Al-Hajj et al., 2003a) and then rapidly emerging investigations on cancer stem cells (CSC), in numerous other solid tumours including glioblastoma (Singh et al., 2004), colorectal (Ricci-Vitiani et al., 2007), liver (Ma et al., 2007), and pancreatic cancer (Li et al., 2007a, Hermann et al., 2007a).

Specific features including self-renew capacity, exclusively tumourigenic in vivo, characterize CSCs, including the capacity of producing all the cancer cell lineages within a tumour (Hermann et al., 2007a, Li et al., 2007b).

The most clinical relevance of CSCs is the intrinsic resistance to standard chemotherapies compared to the their more differentiated progeny (Maugeri-Sacca et al.). Specifically, during treatment, while more differentiated cells regularly respond to chemotherapy, CSCs evade the effect of the chemotherapy, and upon termination of treatment, can again give rise to more differentiated progenies, recapitulating the heterogeneity of the tumour **(Figure I6)**.

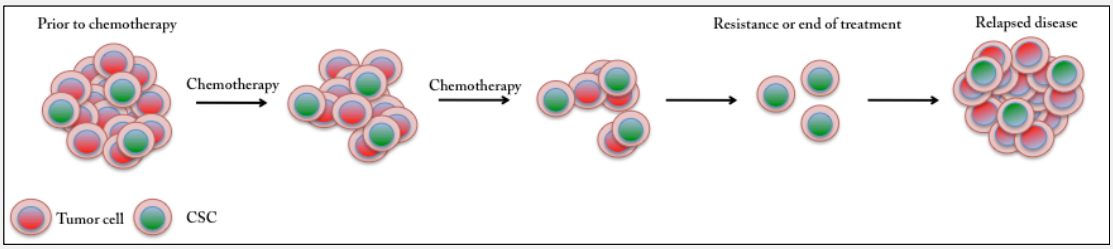



**Figure I6**. During treatment, chemotherapy is able to kill more differentiated cells, while cancer stem cells are not affected by chemotherapy recapitulating the tumour heterogeneity upon treatment cessation.

The mechanisms underlying chemoresistance can be schematically subdivided:

- Proficient DNA repair machinery that preserve of the genetic code from exogenous or endogenous injuries maintaining normal cellular function. DNA sensor and repair pathways act in concert with apoptotic signaling to favors cancer stem cell survival

- ABC drug transporters actively extrude from cancer stem cells a variety of structurally and functionally unrelated drugs of natural origin
- Cell quiescence ensures cancer stem cells do not exhaust their proliferative potential and allow an efficient DNA damage repair and reenter the cell cycle. Quiescent CSCs are mostly spared by chemotherapy-induced cytotoxicity (because affecting the more proliferative cells) and are therefore capable of reconstituting the original tumour.

It is worth some efforts to develop chemotherapy enhancing agents aimed at eliminating CSCs must take safety issues into account to avoid, or at least minimize, the inhibition of crucial mechanisms for normal stem cells.

## 3.1 Markers for the identification of pancreatic cancer stem cells

The first evidence for the existence of CSCs in pancreatic cancer was provided by Li et al. (Li et al., 2007b), in which they identified a highly tumourigenic $CD44^+CD24^+EpCAM^+$ subpopulation of cells using immunocompromised mice xenografted with primary human pancreatic adenocarcinomas. Only these cells were able to form tumours at low numbers and only these cells displayed typical stem cell features like self-renewal, activation of developmental signaling pathways, generation of differentiated progeny and the ability to recapitulate the phenotype of the parental tumour from which they were derived. Unfortunately, it should be noted that in this first study, CSCs were compared to their triple-negative counterparts ($CD44^-CD24^-EpCAM^-$). Since EpCAM identifies epithelial cells within the tumour, it is possible that their EpCAM negative cells represented non-epithelial inflammatory stromal and vascular cells, or cells of mouse origin. Apparently, the finding that tumourigenicity in pancreatic cancer is confined to $CD24^+$ cells is in stark contrast to the original findings in breast cancer, where only $CD24^-$ cells were tumourigenic. In a second study, Hermann et al. (Hermann et al., 2007a) showed that CD133 in pancreatic cancer cell lines and primary pancreatic cancers also reproducibly discriminates for cells with capacity for self-renewal, sphere formation, and, most importantly, in vivo tumourigenicity capability during serial passaging *in vivo*. Not surprisingly, $CD133^+$ cells show some overlap with the $CD44^+CD24^+EpCAM^+$ subpopulation. More recently, additional markers have also been

associated with pancreatic CSC: ALDH-1 has been shown to label tumourigenic cells in pancreatic and breast cancer (Feldmann et al., 2007, Jimeno et al., 2009, Rasheed et al., 2010), although more recent data suggest an abundant expression of ALDH-1 in normal pancreas tissue (Deng et al., 2010), which may compromise the specificity of ALDH-1 as a marker for pancreatic CSCs. Indeed, ALDH-1 can be used for tumours whose normal tissue expression of ALDH-1 is limited or restricted, such as breast, lung, ovarian or colorectal tumours, or for circulating CSCs. **(Table I2).** Since cell surface markers merely enrich for CSC populations, and therefore their use is controversial, functional assays like sphere formation capacity in vitro, and tumourigenicity in vivo, are becoming even more important for the identification of CSC.

| Pancreatic Ductal Adenocarcinoma | Markers | Reference |
|---|---|---|
| Tumor-initiating population | $EpCAM^+$ $CD44^+$ $CD24^+$ | (Li et al., 2007a) |
| | CD133 | (Hermann et al., 2007a) |
| | ALDH-1 | (Feldmann et al., 2007, Jimeno et al., 2009, Rasheed et al.) |
| | Side Population/ABCG2 | (Kabashima et al., 2009) |
| Migrating cancer stem cells | CD133+CXCR4+ | (Hermann et al., 2007a) |

**Table I2.** Cancer Stem cell markers for pancreatic cancer. *Adapted from* (Hermann et al.)

## 3.2 Migrating pancreatic cancer stem cells and metastasis

Metastasis is the major cause of death in pancreatic cancer patients and currently there is no effective treatment available for this deadly disease. Increasing evidences suggest that not all cells within a tumour possess migrating capability; only a small subset of cells is directed through lymphatic or blood vessels towards specific secondary sites to form metastases. In order to be able to establish secondary lesions, the migrating cells would require similar features to the cells initiating the primary tumour. For this reason CSC were proposed to represent the only cell population capable of spreading and giving rise to metastases. Indeed,

Hermann et al. for the first time identified two distinct subsets of CSC based on the expression of the chemokine receptor CXCR4 in pancreatic cancer (Hermann et al., 2007a). CXCR4 is a chemokine receptor responding to chemotactic gradients of its specific ligand SDF-1 that was originally found to be responsible for leukocyte and hematopoietic progenitor cell homing. Emerging evidence suggests that CXCR4 plays a pivotal role in the metastatic process of different tumour entities towards a gradient of SDF-1, which is highly expressed in secondary sites usually associated with metastasis (liver, bone marrow) (Teicher and Fricker). Hermann et al. identified a "stationary" population expressing CD133, but not CXCR4, which is responsible for the initiation and maintenance of the primary tumour, and a "migrating" and highly metastatic population characterized by co-expression of CD133 and CXCR4. Consequently, pharmacological inhibition of the CXCR4 receptor by AMD3100 prevented the metastatic activity of purified CSC. $CD133^{+}CXCR4^{+}$ CSCs were also found in patients with lymph node metastasis (pN1+), demonstrating a close clinical correlation between migrating CSC and advanced disease **(Figure I7)**.

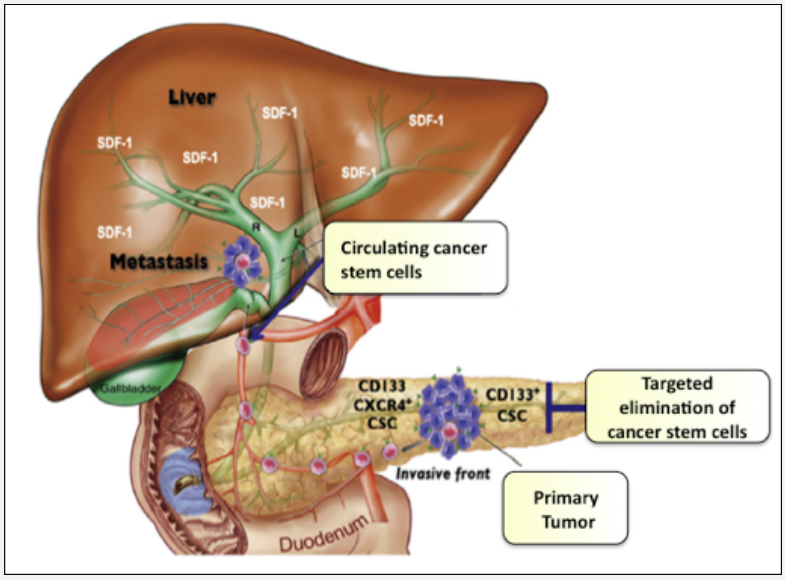



**Figure I7**. A subpopulation of migrating cancer stem cells, identified by CD133+CXCR4+ is responsible for metastasis. Detection of these circulating CSC could serve as prognostic and therapeutic biomarker. *Adapted from (Lonardo et al., 2010)*

CSC in primary tumours may acquire a migrating phenotype through epithelial-mesenchymal-transition (EMT), a complex process that involves several kinases and transcription factor that allows the transformation in a mesenchymal phenotype associated with strong migration capacity. Recently, Wellner et al. showed in pancreatic and colon cancers that the EMT-activator ZEB1 represents an important promoter of metastasis by suppressing E-cadherin and at the same time the stem cell phenotype was maintained by suppression of miR-200 family members that usually target stem cell factors such as Sox2 and Klf4 (Wellner et al., 2009). Taken together, these results suggest that in pancreatic cancer the metastatic process is not random, but rather regulated by specific mechanisms related to the expression of adhesion molecules, chemokine receptors like (i.e. CXCR4) and their respective ligands in secondary organs.

## 3.3 Targeting pancreatic cancer stem cells

Conventional anticancer therapy is directed to rapidly proliferating and differentiated cells that represent the bulk tumour, while sparing the undifferentiated CSCs. Such strategies often have limited efficacy because of intrinsic or acquired drug resistance and/or resistance to ionizing radiation (Frank et al.). While the majority of the cells undergo apoptosis after treatment with gemcitabine, the $CD133^+$ CSCs are able to block the cell cycle in order to have more time to repair the DNA damage; as soon the treatment is withdrawn, these cells proliferate again producing the relapse of the tumour (Hermann et al., 2007a, Jimeno et al., 2009). It is very important to understand the mechanism of chemoresistance in order to find specific therapy in combination with standard chemotherapy. Mechanisms of therapy resistance include increased recognition and repair of DNA damaged (Al-Assar et al., 2011), altered cell cycle checkpoint control and quiescence (Dean et al., 2005), enhanced anti-apoptotic mechanisms (Visvader and Lindeman, 2008), and reduced drug accumulation as a result of increased expression of ABC transporters that efflux drugs (Goodell et al., 1996). These findings provide a rationale for the development of therapeutic strategies directed at targeting relevant molecular pathways in CSCs, or trying to force CSCs into a more differentiated state. Therapeutic approach able to target both CSCs and cancer bulk populations might prove most effective for tumour eradication and prevent relapse.

Nevertheless it is important to recognize that the CSC phenotype can display interindividual variability in particular tumours and that CSC-directed therapies might have limitations with regard to targeting every CSC in all patients (Xia et al., 2012). Some examples of CSC-specific therapeutic targets and therapies are described below.

1) The Sonic Hedgehog (Shh) is a secreted protein that inhibits the transmembrane receptor Patched, which leads to an activation of the Smoothened receptor that activates the transcription of the Gli protein family target genes. Shh pathway plays a critical role in embryonic development of the pancreas (Ingham and McMahon, 2001, Ruiz i Altaba et al., 2002), and more recently in the progression and maintenance of pancreatic cancer and CSCs (Bailey et al., 2009, Morton et al., 2007). Transgenic mice with overexpressing Shh in the pancreatic endoderm developed pancreatic intraepithelial neoplasia (PanIN) and the accumulation of genetic Kras mutations demonstrating that Shh is an early mediator of PC tumourigenesis. Additionally, inhibition of Hedgehog signaling pathway by Cyclopamine retarded PC cell growth and induced apoptosis in vitro and in vivo (Thayer et al., 2003). More recently, inhibition of Shh pathway in mouse model of pancreatic cancer has shown to reduce the stroma content leading to a better delivery of chemotherapy improving survival (Olive et al., 2009). Recently, has been shown that pancreatic CSCs possess an increase activation of the Shh signaling pathway (Dembinski and Krauss, 2010), suggesting that inhibition of the Shh pathway could improve the outcomes of patients by elimination of CSCs. In a more preclinical scenario, it was demonstrate that only a triple combination therapy of Shh inhibitor (cyclopamine), the mTOR inhibitor (rapamycin), and Gemcitabine was capable to target CSCs in primary human pancreatic cancer cells (i.e. CD133+ cells), as shown by abrogation of the tumourigenic potential of cells that were pre-treated *ex vivo* with the triple therapy (Mueller et al., 2009). Even though these preclinical studies looks promising in terms of antitumour effect, unfortunately, advanced PDAC patients in clinical trials showed no improvement in median survival when treated with inhibitors of hedgehog pathway. Different clinical trial designs are needed to better target PDAC cancer and stroma. (Hidalgo and Von Hoff, 2012).

2) mTOR signalling: The mammalian Target Of Rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase, that exerts its functions through phosphorylation of two

major downstream targets, the ribosomal protein S6 kinases and the eukaryotic translation initiation factor 4E-binding protein 1 playing a pivotal roles in many cellular processes including cell growth, cell division, cell cycle progression, and cell metabolism (Inoki et al., 2005). Recently, the mTOR pathway has been reported to be involved in maintaining pancreatic CSCs in particular in CD133+ population within PDAC tumours. However, inhibition of the mTOR pathway by rapamycin resulted in a significant decreased of CD133 population, as also observed for Shh pathway, was not efficient to completely eliminate the CSC pool (Mueller et al., 2009).

3) Notch pathway: The notch pathway plays a pivotal role in cell-cell communication, embryogenesis, cellular homeostasis, cellular differentiation, apoptosis, and stem cell renewal. Four Notch receptors (Notch1–4) and five Notch ligands (Delta-like 1, 3, 4 and Jagged-1, -2) have been discovered: when a Notch ligand binds to an adjacent Notch receptor, Notch will be cleaved by multiple enzymes including γ-secretase, leading to release of the active Notch fragment and activation of Notch target genes including Akt, VEGF, mTOR and NF-kB (Ranganathan et al., 2011). Thus, Notch inhibition has been described to have an anti-proliferative effect or enhancement of apoptotic activity (Hassan et al., 2013). Fan et al. in glioblastoma indicated that inhibition of Notch pathway by use of an inhibitor of γ-secretase (GSI-18) was also capable of significantly reducing the $CD133^{+}$ $Notch^{+}$ cell population, leading to the depletion of medulloblastoma side population cells and tumourigenicity capacity *in* (Fan et al., 2006). These findings might be also applicable to pancreatic CSCs, as Notch 2 has also been implicated in pancreatic cancer progression (Mazur et al., 2010). Another study by Tuveson et al. demonstrated in a mouse model for pancreatic cancer, that the gamma secretase inhibitor MRK003 inhibited Notch signalling in advance PDA but was not efficient to prolong survival of the mice. Only the combination therapy of MRK003 and Gemcitabine was capable to extend the lifespan (Cook et al., 2012).

4) Nodal/ActivinA pathway: Nodal and Activin are members of TGF-ß family and have been shown to play an important role in human embryonic stem cell maintenance through binding to Activin-like type I (Alk4 and Alk7) or II receptors (ActRIIA and ActRIIB) on the surface of the cells, which leads to the phosphorylation of Smad proteins including Smad2 and Smad3 and the formation of a complex with Smad4 that migrate to the nucleus and activate

gene expression including Nanog and Oct4 (Minchiotti, 2005). More recently has been shown that Nodal/Activin is strongly expressed in pancreatic CSCs and are essential for the self-renewal capacity and "stemness" of pancreatic CSCs (Lonardo et al., 2011). Moreover also pancreatic stellate cells, which are present in the tumour stroma and serve as a niche for CSCs, expressed Nodal/Activin (Lonardo et al., 2012). Using primary pancreatic cancer cells, the authors showed that the inhibition of Nodal/Activin pathway in CSCs using SB431542, a specific inhibitor of the Nodal/Activin receptor Alk4, recombinant Lefty, a specific endogenous Nodal inhibitor, or genetic knockdown of Nodal/Alk4 and Smad4 using shRNA technology, abrogated their self-renewal capacity and tumourigenicity, and reversed the resistance of CSCs to gemcitabine. Importantly, the Nodal/Activin pathway lacks activity in adult tissues (e.g. normal pancreas) making it a realistic and plausible therapeutic target in patients with pancreatic cancer.

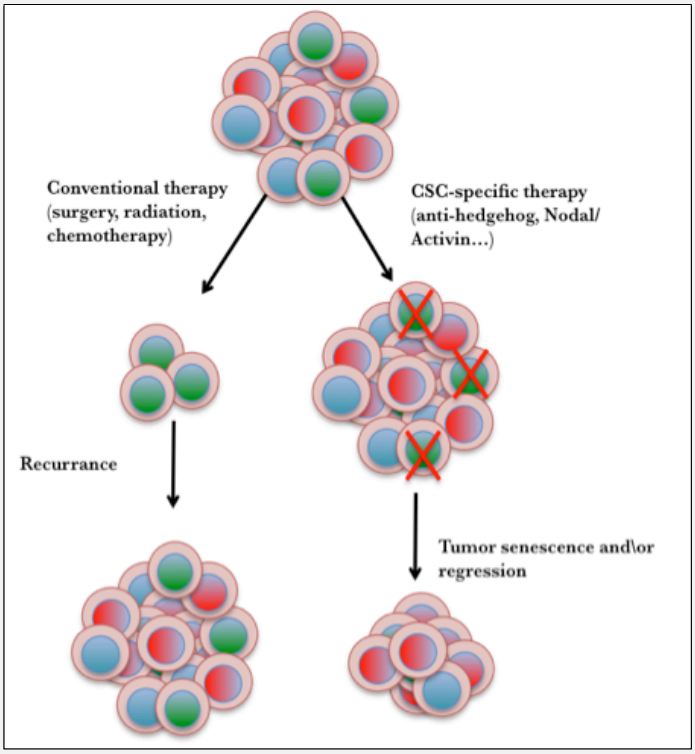



**Figure I8**. Conventional therapies eliminates the bulk of the tumour but does not affect CSCs, resulting in a tumour relapsed. CSC target therapies, will kill or differentiate the CSCs, resulting in a loss of tumour initiating cells that will conduce to a regression. *Adapted from (Ebben et al., 2010).*(Green nuclei=CSC, blue and red nuclei=tumour cell)

In conclusion, developing new therapeutic strategies that include targeting of CSC bear great potential, but, given the enourmous intertumoural heterogeneity these efforts should also consider personalized approaches where a panel of available drugs is tested for individual response (**Figure I8**).

## 4. MicroRNA

MicroRNAs belong to a class of non-coding RNAs that play key roles in the regulation of gene expression at post-transcriptional level (Bartel, 2004). MicroRNAs are generally transcribed by RNA polymerase II as large primary transcripts (pri-microRNA) that are processed by a protein complex containing the RNase III enzyme Drosha and the double-stranded-RNA-binding protein, Pasha (also known as DGCR8) to form an approximately 70 nucleotide precursor microRNA (pre-microRNA) with a stem-loop structure (Lee et al., 2002, Esquela-Kerscher and Slack, 2006) **(Figure I9)**.

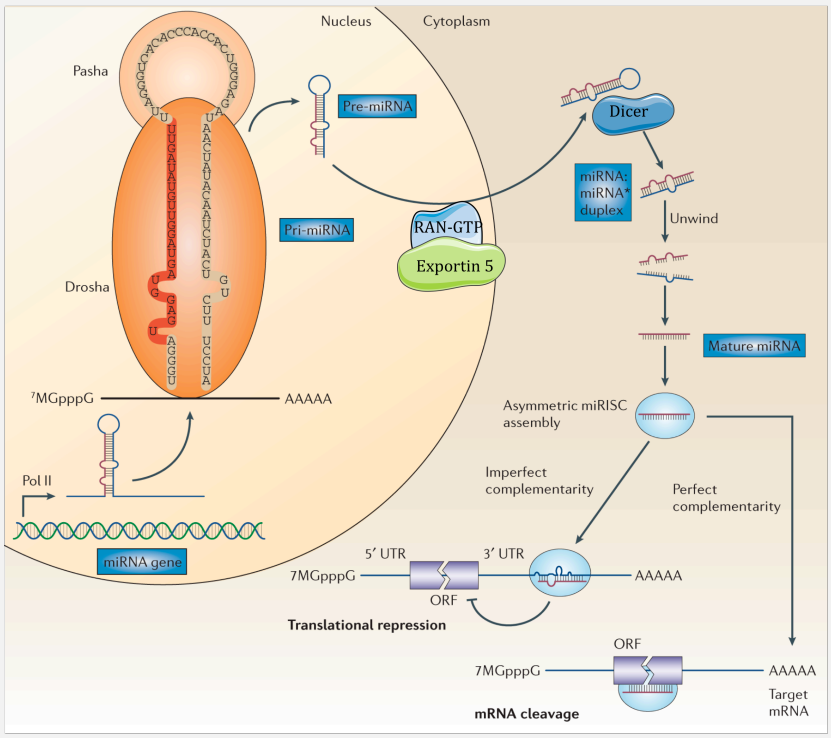



**Figure I9**. The miRNA biogenesis pathway in vertebrate cells. *Adapted from (Esquela-Kerscher and Slack, 2006)*

This precursor is subsequently transported to the cytoplasm by the RAN GTP-dependent transporter exportin 5 (Kim, 2004) where it is processed by a second RNase III enzyme, DICER, to form a mature microRNA of approximately 22 nucleotides (Hutvagner et al., 2001). The mature miRNA:miRNA* duplex is then incorporated into a ribonuclear particle to

form the RNA-induced silencing complex, RISC, which negatively regulates its target genes. miRNAs are able to recognize their target mRNAs by as little as 6-8 nucleotides (the seed region) at the 3' untranslated regions UTR end of mRNA. Based on the complementarity between the miRNA and the target mRNA, there are two ways of inhibition: if the complementarity is perfect, the products follow the RNA-mediated interference (RNAi) pathway in which mRNA transcripts are degraded by ribonucleases (Hannon, 2002), while there is no degradation if the complementary is imperfect affecting only the protein target, but not the mRNA expression (Pillai et al., 2005). Since most target sites on the mRNA have only partial base complementarity with their corresponding microRNA, individual microRNAs may target as many as 100 different mRNAs. Moreover, individual mRNAs may contain multiple binding sites for different microRNAs, resulting in a complex regulatory network. In the same way, a single miRNA might bind as many as 200 gene targets and that these targets can be diverse in their function; so, miRNAs potentially control the expression 30% of all human protein-encoding genes.

In humans, approximately one third of miRNAs are organized in clusters. A given cluster is a single transcriptional unit, suggesting a coordinated regulation of miRNAs in the cluster. In silico analysis revealed that more than half of the clusters contain two or more miRNAs of similar sequence (Lee and Dutta, 2009). Members of the same cluster are similar in sequence, but can exert different and opposing biological effects. However, it is worth noting that all the miRNAs from a single transcriptional cluster are not expressed at equal levels, suggesting that miRNAs are also regulated post-transcriptionally.

## 4.1 MicroRNAs and Cancer

MicroRNAs have been shown to be involved in a wide range of biological processes such as cell cycle control, apoptosis and several developmental, physiological and pathological processes including stem cell differentiation, haematopoiesis, hypoxia and aging. In addition, highly tissue-specific expression and distinct temporal expression patterns during embryogenesis suggest that microRNAs play a key role in the differentiation and maintenance of tissue identity. MicroRNA has been also associated with cancer where can function as oncogene or tumour suppressor depending on the specific tumour, cell population and

microenviroment composition (Calin et al., 2004). Recently has been showed that about 50% of human miRNAs are located in areas of the genome containing fragile sites, frequently associated with cancer. This indicates that miRNAs might have a crucial function in cancer progression. For example, *mir-125b-1* is located in a fragile site on chromosome 11q24, which is deleted in a subset of patients with breast, lung, ovarian and cervical cancer (Calin et al., 2004). In some other cases there is an aberrant expression of miRNA that act as oncogene, actively contributing to cancer development. There are several mechanisms that lead to an aberrant expression of miRNAs:

- Genomic abnormality such as deletion, amplification, translocation. The first example of tumour suppressors miRNA is provided by Calin *et al.* in which identified a genomic deletions within the 13q14 locus in more than 65% of B-cell chronic lymphocytic leukemia (CLL) cases, as well as in 50% of mantle cell lymphomas, 16–40% of multiple myelomas and 60% of prostate cancers. They hypothesized that in that 30kb region must reside a tumour suppressor gene and they identified two clustered miRNA genes, *mir-15a* and *mir-16-1* (Calin et al., 2002). Further studies showed that miR-15a and miR-16-1 negatively regulate *BCL2*, which is an anti-apoptotic gene that is often over-expressed in many types of human cancers, including leukaemias and lymphomas (Cimmino et al., 2005).

- Epigenetic factors can influence the expression of miRNA through hypermethylation of CpG islands located very close of several miRNAs (79). Treatment of cancer cells with HDAC (histone deacetylase) inhibitor or with 5-aza-2′-deoxycytidine can up-regulate specific miRNA (Scott et al., 2006). One example is miR-124a that is hypermethylated is tumour-type specific manner in colorectal tumours, while no methylation was seen in neuroblastoma where its expression is very high (Lujambio et al., 2007).

- Regulation of miRNA processing steps is important to determine miRNA expression level. Although miRNAs from a genomic cluster are expressed from a common pri-miRNA, the levels of individual miRNAs in the cluster are not necessarily coordinated (Lu et al., 2007). Moreover, miRNAs often exhibit a discrepancy in expression of the

mature form compared with its precursor due to mutations or alterations in expression level of several protein involved in miRNA biogenesis (Lee et al., 2008, Obernosterer et al., 2006). The altered expression levels of Dicer or Drosha have been demonstrated in several cancers (Chiosea et al., 2007, Muralidhar et al., 2007) likely due to change in copy number of the gene. Specifically Dicer has been implicated in heterochromatin maintenance and centromeric silencing, reduced protein levels might directly result in genomic instability and lead to tumour formation (Fukagawa et al., 2004). Moreover, the Argonaute proteins, which are crucial components of the RISC complex that direct both short interfering (siRNA)- and miRNA-mediated gene regulation, have also been associated with various cancers (Carmell et al., 2002).

The majorities of miRNAs are expressed in a tissue specific manner and are different among cancers from various tissue origins. There are also some miRNAs that appear to be frequently deregulated in many cancers, suggesting that these miRNAs regulate fundamental processes during tumourigenesis. One example is the miR-17-92 cluster, located at chr13q31.3 in humans and composed of six miRNAs (mir-17, -18a, -19a, -20a, -19b-1, and -92a-1). The miR-17-92 cluster has been identified as potential oncogene for the first time in B-cell lymphoma where forced expression of the miR-17-92 cluster in transgenic mice overexpressing c-Myc oncogene significantly accelerated the oncogenic process (He et al., 2005). Specifically, c-Myc is able to bind the genomic region upstream of the miR-17-92 cluster and activates its expression (O'Donnell et al., 2005) together with E2F1 forming a positive feedback loop because E2F1 is a direct target of miR-17 and -20a (Sylvestre et al., 2007). During proliferation, c-Myc and E2Fs turn on the miR-17-92 cluster that on the other way represses E2Fs, thereby preventing the uncontrolled amplification of the positive feedback loop between E2Fs and c-Myc: cycling cells will have elevated steady state levels of miR-17–92 due to the periodic burst of E2F activity during S phase, while quiescent cells will have reduced miR-17–92 levels (Woods et al., 2007). Consistent with its oncogenic role, the miR-17-92 cluster has been found up-regulated in a variety of cancers including lymphomas (Rinaldi et al., 2007), lung cancers (Hayashita et al., 2005), and others (Volinia et al., 2006) through amplification of chr13q31 locus (Ota et al., 2004). Recent evidences show that the miR-17-92 cluster, paradoxically could act as a tumour suppressor in some circumstances. Loss of heterozygosity at 13q12-q13 is associated with multiple tumour progression and poor

prognosis, including breast cancer, squamous cell carcinoma of the larynx, retinoblastoma, hepatocellular carcinoma and nasopharyngeal carcinoma (Xiang and Wu, 2010). Since c-Myc and E2Fs induces expression of the miR-17-92 cluster during proliferation of cells, the same cluster, which is able to down-regulates E2Fs, may serve as a brake on excessive proliferation acting as tumour suppressor. The *miR-17-5p* exerts its role of tumour suppressor in breast cancer cells by repressing the expression of amplified in breast cancer 1 (AIB1). Over-expression or down-regulation of the *miR-17-5p* could suppress or promote breast cancer cell proliferation, respectively (Hossain et al., 2006). Recently, has been shown that over-expression of miR-17-92 reduce tumourigenicity and induce apoptosis in gastrointestinal stromal tumour (GIST) by down-regulation of KIT and ETV1 (Gits et al., 2013).

## 4.1 MicroRNAs and Cancer Stem Cells

Recent studies suggest that miRNAs are able to regulate not only self-renewal and differentiation, but also important signalling pathways involved in the regulation of CSCs (Bao et al., 2010). Breast cancer stem cells (BCSCs) are the best characterized system in solid tumours and can be identified using surface markers $CD44^{+}CD24^{-/lo}$ (Al-Hajj et al., 2003b) or Aldefluor assays (Ginestier et al., 2007). One of the first studies of miRNA regulation of CSCs shows that let-7 was significantly reduced in BCSCs (Yu et al., 2007). Based on this study, has been demonstrated that let-7 regulated self-renewal and differentiation, mammosphere formation, tumour formation, and metastasis in non-obese diabetic (NOD)/severe combined immunodeficient mice (SCID) by targeting H-RAS and HMGA2. Subsequently, miR-30 and miR-200 family were also found to be one of the miRNAs markedly reduced in BCSCs and to negatively modulate the stemness of BCSCs, suggesting that multiple miRNAs may regulate CSC properties (Yu et al., 2010, Shimono et al., 2009). The miR-200 family is also important for the maintenance of pancreatic cancer stem cells (PaCSCs) because is able to repress the expression of stem cell transcription factors such as Sox2 and Klf4. Furthermore, the members of this family (miR-141, miR-200a, b, c and miR-429) can suppress the transcriptional repressor ZEB1, important for EMT and metastasis (Wellner et al., 2009, Spaderna et al., 2008) linking CSCs, miRNA and EMT process. In fact ZEB1 is expressed at the invasive front of pancreatic cancer and highly correlates with the

expression of miR-200 family members. Moreover the knockdown of ZEB1 resulted in an epithelial transition in pancreatic cancer cell lines with mesenchymal phenotype (MiaPaCa2 and Panc1) and affected CSCs features such as spheres formation in culture and chemoresistance. Another CSC-regulating miRNA in pancreatic cancer is the miR-34 that is directly regulated by p53 and has been identified as tumour suppressor miRNA (Tarasov et al., 2007, Chang et al., 2007). Interestingly, miR-34 regulates Notch pathway and Bcl-2 that are important for CSCs maintenance. Restoration of miR-34 reduce CSC population, inhibits sphere formation *in vitro* and tumour formation *in vivo* (Ji et al., 2009). These results suggest that miRNA can be responsible for important pathway involved in CSCs biology and metastasis.

The increasing knowledge of miRNA biology can open to new approaches in cancer diagnosis and therapy. Large-scale miRNA expression profiling of tumours versus normal tissue are useful to classify cancers and to define miRNA markers that might predict favourable prognosis.

The administration of synthetic anti-sense oligonucleotides that encode sequences that are complementary to mature oncogenic miRNAs — termed anti-miRNA oligonucleotides AMOs) — might inactivate miRNAs in tumours and slow their growth. Formulations including conjugation with cholesterol improve stability, efficiency and toxicity in pre-clinical settings. Conversely, techniques to overexpress miRNAs by lentiviral approach or liposomal miR-mimics that function as tumour suppressors can be used to treat specific tumour types. Efficient over-expression of miR-34a in prostate cancer stem cells was able to reduce tumourigenicity and metastasis in a pre-clinical setting (Liu et al., 2011). Additional studies to improve the efficiency of these methods are needed before miRNA treatments can move from the laboratory bench to the bedside.

# OBJECTIVES

Pancreatic cancer is one of the most aggressive types of cancer due to intrinsic chemoresistance and early metastasis. Recently it has been demonstrated that pancreatic cancer contains a subpopulation of CSCs expressing CD133 that is essential for maintenance of tumour progression, metastatic spread and for the relapse after standard chemotherapeutic treatment (Hermann et al., 2007b). However, the mechanisms that govern self-renewal and resistance to chemotherapy remain poorly understood. Recently, it has been demonstrated that Nodal/Activin signalling is one of the master regulators of self-renewal in pancreatic cancer stem cells (Lonardo et al., 2011). Others mechanisms relevant for CSCs biology include epigenetic regulation through miRNAs, which have already been implicated in the regulation of normal stem cells as well as CSCs in other cancers (Croce and Calin, 2005, Melton et al., 2010, Yu et al., 2007, Shimono et al., 2009). Therefore, advancing our understanding about miRNA regulation of drug resistance and self-renewal in pancreatic CSCs could lead to novel and more effective therapeutic strategies for patients with pancreatic ductal adenocarcinoma.

In the present PhD thesis project, we are aiming to:

1. Identify modulated miRNA using high-throughput expression analysis of primary human PDAC cells resistant to chemotherapy and enriched for CSCs;
2. Validate miRNAs significantly downregulated in chemo-resistant CSCs;
3. Define the biological effect of the miRNA identified using loss of function and gain of function approaches;
4. Identify miRNA targeted proteins and validate their functional relevance in CSCs biology.

# OBJETIVOS

El cáncer de páncreas es uno de los tipos de cánceres mas agreviso debido a su característica quimioresistente y pronta metástasis.. Recientemente, ha sido demonstrado que el cáncer de páncreas contiene una subpoblación de CSCs que expresan CD133. Se ha demostrado que el marcador CD133 es esencial en el mantenimineto y progresión del tumor, asi como está implicado en metástasis y recurriencia después del tratamiento con quimioterapia estándard (Hermann et al., 2007b). Sin embargo, el mecanismo que conduce a la auto-replicación y resistencia a la quimioterapia todavía queda por entender. Recientemente, se ha demostrado que la via de señalización de Nodal/Activin es uno de las principales reguladores de la capacidad de auto-replicación en células troncales de cáncer (Lonardo et al., 2011). Otros mecanismos relevantes en la biología de las CSCs incluye la regulación epigenética a través de los miRNAs, los cuales han sido ya impliacos en la regulación de las células troncales normales , como también en otras CSCs de otros tipos de cánceres (Croce and Calin, 2005, Melton et al., 2010, Yu et al., 2007, Shimono et al., 2009). Por lo tanto, si progresamos en el entendimiento de la regulación de los miRNA en la capacidad de auto-replicación y resistencia a quimioterapéuticos de las células troncales de cáncer, podría ayudar a conseguir nuevas estrategias para poder obtener terapias mas eficientes para estos pacientes con adenocarcinoma pancreático.

En el presente proyecto de tesis doctoral, nuestro objetivo es:

1. Identificar miRNA modulados usando análisis de expresión de alto contenido de células primarias de adenocarcinoma pancreático que son resistentes a la quimioterapia y están enriquecidas en CSCs
2. Validar los miRNAs que tienen una expresión significativamente menor en las CSCs que son quimioresistentes.
3. Definir los efectos biológicos the los miRNA identificados usando la técnica de perdida y ganancia de funcionalidad;
4. Identificar proteínas de los miRNA y validar su relevancia funcional en la biología de las CSCs.

# Materials and Methods

# 1. MICE

## 1.1 Study approval

Mice were housed in the CNIO's animal facility in accordance with institutional policies and federal guidelines. Animal treatments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain). Human pancreatic tumours were obtained with written informed consent and after approval from the Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain).

## 1.2 Xenograft

PDAC xenografts, generated from patient-derived samples, were kindly obtained from Manuel Hidalgo's group (CNIO, Spain). Primary tumours were minced into small fragments and then implanted subcutaneously, in duplicate, in 4 to 5 nude mice (NU-*Foxn1*$^{nu}$; Charles River, Wilmington, MA, USA). Once tumours reached 1cm$^3$, tumours were resected, minced and re-implanted in another set of female nude mice, following the protocol described in Rubio- Viqueira et al. (Rubio-Viqueira et al., 2006), and illustrated in **Figure M&M1.**

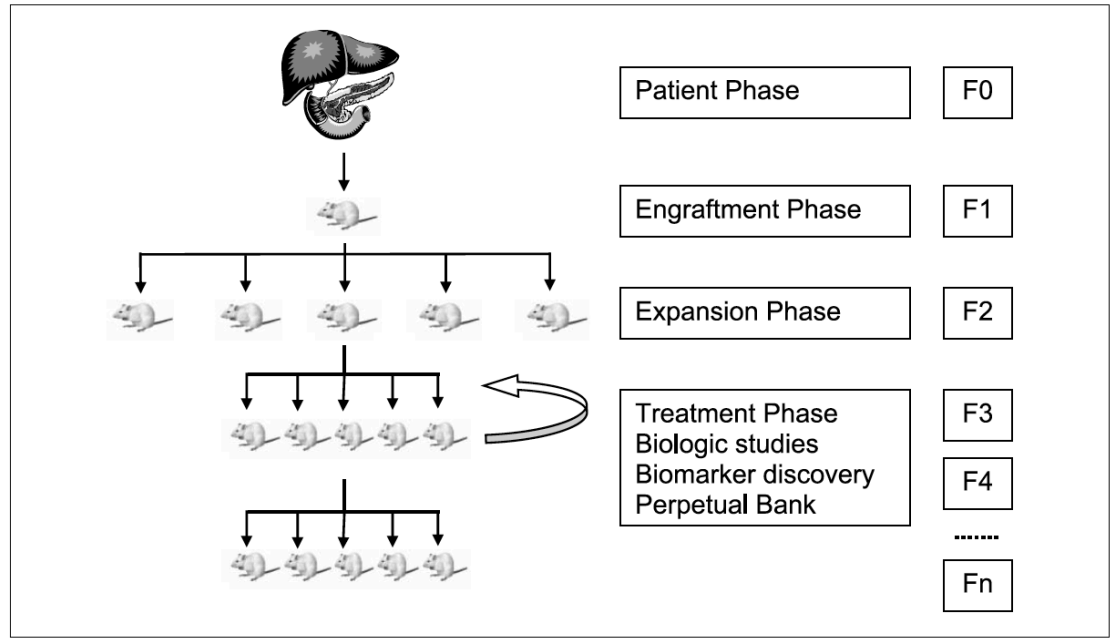



**Figure M&M1**. Xenograft study scheme. Tumour samples are implanted in F1 generation mice and then expanded in a cohort of nude mice. *Adapted from Rubio-Viqueira et al., 2006.*

### 1.3 Mice treatments

Four to six week-old female nude mice were used as recipients for primary human xenograft transplantations and mCherry or GFP or ZsGreen sorted primary cells (see below). Upon reaching a volume of 200mm$^3$, mice were assigned to the following treatment groups: Control, Abraxane (Celgene, NJ, USA) 50mg/kg twice weekly (i.v) during 15 days and Gemcitabine (Eli Lilly, IN, USA) 125mg/kg twice weekly (i.p.) during 40 days.

### 1.4 In vivo tumourigenicity and metastasis assays

Primary pancreatic cancer cells were sorted for mCherry or GFP depending on the Lenti-vector used as detailed below. For tumourigenicity assays, serial dilutions ($10^4$ $10^3$ $10^2$) of single-cells resuspended in Matrigel™ (BD Bioscience, Heidelberg, Germany) were subcutaneously injected into right and left flank of female nude mice. For serial in vivo transplantation the tumours were digested, sorted for GFP or ZsGreen and injected again in the same number of cells ($10^4$). For metastasis assays, $10^4$ FACSorted mCherry positive, GFP or ZsGreen positive and negative cells, were resuspended in 1X PBS and intrasplenically injected into NSG mice as previously described (Sainz et al., 2012).

## 2. CELL CULTURE

### 2.1 Primary human cancer cells.

Primary pancreatic cancer tumours were minced, mechanically (gentleMACS Dissociator; Miltenyi, Bergisch-Gladbach, Germany) and enzymatically digested with collagenase for 60 min at 37°C followed by a centrifugation for 5 min at 1200 rpm (Mueller et al., 2009) (Stem Cell Technologies, Vancouver, BC). Cell pellets were resuspended and cultured in RPMI (Invitrogen, Alcobendas, Spain) supplemented with 10% FBS and 50 units/ml pen/strep.

### 2.2 Sphere formation assay

Spheres were generated by culturing ~2×$10^4$ pancreatic cancer cells in Ultra-Low attachment plates (Corning, USA) in suspension in serum-free DMEM/F12 supplemented with B27 (1:50, Invitrogen, Alcobendas, Spain), 20 ng/ml bFGF and 50 units/ml pen/strep for a total of 7 days, allowing spheres to reach a size of >75μm. For serial passaging, 7-day-old spheres

were harvested using 40µm cell strainers, dissociated into single cells, and then re-cultured for 7 additional days as previously described (Lonardo et al.).

## 2.3 PKH26 assay.

Human primary pancreatic cancer cells were labelled with PKH26, a red fluorescent cell membrane labelling dye (Sigma), according to manufacturer's instructions. Every 7 days, cells were harvested and PKH26+ cells were determined using a FACS Canto II (BD) for a total of 4 weeks.

## 2.4 Cell viability assay

Cells were seeded in 96-well plates (Nalgen Nunc International, Penfield, NY) at a concentration of $10^3$ cells per well in 100µL of complete medium. Cells were incubated for 24 hours after administration of compounds to allow an optimal attachment. Cytoxicity was measured using a sulforhodamine B (SRB)-based cytotoxicity assay as described previously (Limame et al., 2012). The protein absorbance of the viable cells at each concentration is expressed as the relative percentage of absorbance compared to the un-treated control well. Each experiment was carried out with three replicate wells for all conditions tested, and all the experiments were done in triplicate.

## 2.5 Wound-healing assay

Confluent cultures of primary cancer cells seeded in a 6-well plate were scratched using a 1ml pipette tip after overnight starvation. Cells were washed twice with PBS to remove cell debris and then incubated at 37°C with serum-free in media in the presence or absence of 300ng/ml recombinant human Nodal, 100ng/ml Activin, and 10ng/ml TGF-β1. Migration was evaluated 24 h later by calculating the average size of the wound determined by measuring the size of the wound at three locations ($n$ = 3 wounds per cell/treatment).

## 2.6 Invasion and migration assays

Invasion assays were performed using modified Boyden chambers filled with Matrigel™ (BioCoat®, BD Biosciences). Human primary pancreatic cancer cells were added to the Matrigel™ coated inserts, and 750µl of serum-free medium with 300ng/ml recombinant human Nodal,

100ng/ml Activin, and 10ng/ml TGF-β1 was added to the lower chamber. The assay chambers were incubated for 22h at 37˚C. Invaded cells were fixed in 4% PFA and stained with DAPI. DAPI-positive nuclei were quantified in 10 high-power fields (HPF). Invasion was then calculated as the number of cells in the lower chamber versus total seeded cells (in %).

## 2.7 Plasmid construct and transfection

pCAGA12-luc SMAD4 reporter is a synthetic Smad responsive luciferase reporter vector that was generated cloning 12xCAGA (consensus Smad binding element) into pGL3 basic plasmid (Promega) (Dennler et al., 1998, Savary et al., 2013). Human primary pancreatic cells were plated into 24-well plates and co-transfected with pCAGA12-luc SMAD4 reporter and control Renilla plasmid using Lipofectamine 2000 and after 48 hours Gaussian luciferase and Renilla luciferase were measured. Luciferase activity was plotted as a percentage of the mock transfection.

## 2.8 3' UTR luciferase reporter assays

The GLuc-ALK4-3'-UTR clone and GLuc-Tbx3-3'-UTR clone (GeneCopoeia, Labomics, Nivells, Belgium) and a control 3'UTR-reporter construct were transfected in human embryonic kidney (HEK) 293T into 24-well plates and co-transfected using Lipofectamine 2000 (Invitrogen) with 10 ng of a reporter plasmid containing the 3′ untranslated region (UTR) of ALK4 or Tbx3 inserted downstream of the Gaussian luciferase secreted reporter gene and the secreted alkaline phosphatase tracking gene and 50 nmol/L of miR-17-92 precursor or negative control. Gaussian luciferase and alkaline phosphatase activities were measured by luminescence in conditioned medium 48 hours after transfection using Dual-Light® detection system (Genecopoeia). Luciferase activity was plotted as a percentage of the mock transfection.

## 2.9 Antagomirs

Knockdown of miR-17-92 was achieved *in vitro* and *in vivo* by administering miR-17, 18a, 19a, 19b, 20a, 92 antagomir mix or scrambled control were chemically synthesized as 2 -O-methyloligoribonucleotides by BioSpring (Frankfurt, Germany). The antagomirs are labeled with Cy3 and contain cholesterol that facilitate the entry into the cells and allow tracking them.

## 2.10 Cell treatments

For chemoresistance studies, primary human cell lines were treated with Gemcitabine (100ng/ml) or Abraxane (10uM) for 7 days. Media with corresponding compound was replaced every 48h.

## 3. FLOW CYTOMETRY

### 3.1 Flow cytometry analysis

For flow cytometry analysis, primary pancreatic cells, dissociated cells from spheres cultures or cells obtained from tumour digestions were stained using different combinations of antibodies. The following antibodies were used: anti-hCD133/1-APC or PE (Miltenyi Biotec); hEpCAM-APC, hCD44-APC, hSSEA-1-APC, hCXCR4-APC, mCD45-APC, mCD146-FITC or appropriate isotype-matched control antibodies (all from BD, Heidelberg, Germany). DAPI was used for exclusion of dead cells. Cells were acquired with a FACS CANTO II instrument (BD, Heidelberg, Germany). Data were analysed with FlowJo 9.2 software (Tree Star, Ashland, OR).

### 3.2 FACS sorting

Primary pancreatic cells, dissociated cells from sphere cultures or cells obtained from tumour digestions were adjusted to a concentration of $10^6$ cells/ml in Sorting buffer [1X PBS; 3% FBS (v/v); 3mM EDTA (v/v)]. DAPI was added to exclude dead cells at a concentration of 2mg/ml. Cells were sorted with a FACS Influx instrument (BD, Heidelberg, Germany).

### 3.3 Apoptosis assay

Cancer cells and CSCs were plated at 3x105 cells/well in a 6-well multi-well and cultured in presence of gemcitabine (100ng/ml) for 7 days. Attached and floating cells were collected, resuspended and stained with Annexin V (550474; BD Bioscience) after incubation with Annexin V binding buffer (556454; BD Pharmingen). Cells were then incubated with DAPI.

### 3.4 Cell cycle analysis

Cells were trypsinized, washed in PBS, centrifuged, and pellets were fixed in 200µl of 70% ethanol and stored at -20°C until use. Cells were centrifuged and pellets resuspended in 200µl of PBS, and 10µg/mL of RNAse A was incubated for 1 hour at 37°C. Subsequently, cells were resuspended in propidium iodide solution (0.1% sodium citrate, 0.1% TritonX-100, and

50µg/mL propidium iodide). Cell-cycle analysis was carried out by flow cytometry (FACS-CANTO). Data were analysed by FlowJO software. For the identification of G0 quiescent population, cells were fixed in 100% ethanol at -20°C overnight, washed with PBS twice and stained with Ki67 (BD, Heidelberg, Germany) for 30min at room temperature, followed by an additional wash with PBS. Cells were stained with DAPI to perform cell cycle analyses using a FACS CANTO II (BD) instrument.

## 4. PROTEIN ANALYSIS

### 4.1 Protein extraction and quantification

Cells were harvested in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The cell lysate was centrifuged at 14,000 rpm and supernatant was collected. Protein lysates were quantified using a BCA Protein Assay Reagent kit (Pierce, Thermo Scientific).

### 4.2 Western Blot

Cells were harvested in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). 50µg of protein was resolved by SDS-PAGE and transferred to PVDF membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 1X TBS containing 5% BSA (w/v), 1% chicken albumin (w/v) and 0.1% Tween20 (v/v), incubated with a 1:1000 dilution of antibodies against p21 (2947), p27 (3688), p57 (2557), Cyclin D1 (2926), pSMAD2 (3108) from Cell Signaling or GAPDH (ab8245), Tbx3 (ab58264), Nanog (ab14959) from Abcam overnight at 4ºC, washed 3 times with 1X PBS containing 0.05% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rat or goat anti-mouse antibody (Sigma), and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Amersham, Barcelona, Spain).

# 5. RNA AND MICRORNA ANALYSIS

## 5.1 RNA extraction from tissue or cells

Total RNAs from human primary pancreatic cancer cells or livers of NSG mice were extracted with TRIzol (Life Technologies, Madrid, Spain) according to the manufacturer's instructions.

## 5.2 RT-qPCR

One microgram of total RNA was reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies) using random hexamers. Quantitative real-time PCR was performed with an Applied Biosystems 7500 real-time thermocycler (Applied Biosystems, Alcobendas, Spain) using Fast SYBR Green (Qiagen, Barcelona, Spain) as per the manufacturer's instructions. The list of utilized primers are shown in **Table M&M1**

*Table M&M1- RT-qPCR primers*

| Gene | Primer sense | Primer antisense |
|---|---|---|
| NANOG | TGAACCTCAGCTACAAACAGGTG | AACTGCATGCAGGACTGCAGAG |
| KLF4 | ACCCACACAGGTGAGAAACC | ATGTGTAAGGCGAGGTGGTC |
| SOX2 | AGAACCCCAAGATGCACAAC | CGGGGCCGGTATTTATAATC |
| OCT3/4 | CTTGCTGCAGAAGTGGGTGGAGGAA | CTGCAGTGTGGGTTTCGGGCA |
| NODAL | AGCATGGTTTTGGAGGTGAC | CCTGCGAGAGGTTGGAGTAG |
| ACTIVIN | AAAGCTTCATGTGGGCAAAG | AATCTCGAAGTGCAGCGTCT |
| TGFB1 | CAACAATTCCTGGCGATACCT | CGTTGATGTCCACTTGCAGT |
| SMAD2 | TCCCAGCAGGAATTGAGCCACA | GTTCTGCTGGAGAGCCTGTGTCC |
| SMAD4 | CAGCACCACCCGCCTATGCC | TGGAACACCAATACTCAGGAGCAGG |
| ALK4 | GGAGCGTCTTGTCTTTGGAG | TGCAACAGGATCGACTTGAG |
| TGFBRII | CAACCACCAGGGCATCCA | TCGTGGTCCCAGCACTCA |
| TBX3 | CGGGAAGGCGAATGTTTCCTCCA | GGTCGGCCTTACCAGCCACC |
| CD133 | CAGAGTACAACGCCAAACCA | AAATCACGATGAGGGTCAGC |
| MCHERRY | GCGCCTACAACGTCAACATC | GCGTTCGTACTGTTCCACGA |
| CDKN1A | CCTGTCACTGTCTTGTACCCTTG | AGAAGATCAGCCGGCGTTT |
| CDKN1B | TAATTGGGGCTCCGGCTAACT | TTGCAGGTCGCTTCCTTATTC |
| CDKN1C | TCTGATCTCCGATTTCTTCGC | TGCTGCTACATGAACGGTCC |
| CYCLIN D1 | CGTGGCCTCTAAGATGAAGGA | CGGTGTAGATGCACAGCTTCT |
| HCNT1 | GGTGGCCTGCCTCCTGGATT | AAGCAGCAAGAGCTAGACCCCTCT |
| HCNT3 | CTTTTCTGGAGTACACAGATGCT | CGGCAGGACCTTAAATGCAAA |
| HENT1 | CTCTCAGCCCACCAATGAAAG | CTCAACAGTCACGGCTGGAA |
| HENT2 | TCTCCAACTCTCAGCCCACCAA | CCTGCGATGCTGGACTTGACCT |
| ABCC1 | GGAATACCAGCAACCCCGACTT | TTTTGGTTTTGTTGAGAGGTGTC |
| ABCG2 | TCATGTTAGGATTGAAGCCAAAGGC | TGTGAGATTGACCAACAGACCTGA |
| B-ACTIN | GCGAGCACAGAGCCTCGCCTT | CATCATCCATGGTGAGCTGGCGG |

### 5.3 miRNA microarray

Total RNA concentrations extracted with Trizol were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The miRNA microarray experiments were performed using the Agilent Microarray Platform (Agilent Technology, Foster city, CA) G4471A-025987 miRNA array composed by 866 human miRNAs probes and the Human Gene Expression 4x44K v2 Microarray contain 34,127 Entrez Gene RNAs. 100 ng total RNA was hybridized with the miRNA array and for each miRNA, multiple probes were spotted on the array and the average intensity represent the expression value of the miRNA. In addition, multiple spots were included as negative controls. The arrays were scanned using an Agilent Technology G2565BA scanner and the scanned images were processed using the Feature Extraction software package version 9.5 (Agilent Technology). For the data processing we performed a global normalization via quantile normalization for each set of sample pair and subsequently a pairwise comparison via unpaired t-test and Benjamini Hochberg correction for false discovery rate.

### 5.4 miRNA RT-qPCR

For miRNA analysis, one microgram of total RNA was reverse-transcribed using the NCode VILO miRNA cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). This step adds a polyadenylate tail to the miRNA population within the total RNA samples. The resulting cDNA was subjected to real-time PCR using SYBR Green ER qPCR Mix (Invitrogen). The Universal qPCR Primer was provided in the VILO kit and the forward primer for miR-17, 18a, 19a, 19b, 20a, 92, Snord95 and Snord44 were purchasing from Quiagen.

## 6. IMAGING

### 6.1 Measuring γH2AX foci

Cells were harvested and cytospins were made. Cells were fixed (2% formaldehyde), permeabilised (0.5% Triton/PBS) and labelled with anti-phsopho-γH2AX (Ser139) antibody (Millipore) overnight, 4°C. Cells were washed, labelled with goat anti-mouse-Alexa488 secondary antibody (Invitrogen) and washed again. Slides were mounted in Vectashield

containing DAPI (Vector Laboratories). Images were taken with an Olympus FluoView 1000 laser-scanning microscope (60x/1.4 PlanApo Oil lens). To quantify the number of γH2AX foci per nucleus ImagePro Software was used, a minimum of 50 nuclei per sample were counted.

### 6.2 Immunohistochemistry

For histopathological analysis, FFPE blocks were serially sectioned (3 μm thick) and stained with haematoxylin and eosin (H&E). Additional serial sections were probed with antibodies against dsRed (Clontech, Saint-Germain, France), human cytokeratin 19α (abcam, Cambridge, UK), or in situ hybridization was performed using the Alu probe (Qbiogene, Bath, UK). Following incubation with primary antibodies, samples were incubated with HRP-conjugated secondary antibodies (DAKO, Barcelona, Spain) and positive cells were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+) as a chromogen.

## 7. LENTIVIRUS

To stably overexpress miR-17-92 cluster we used lentiviral particles carrying pre-miR17-92 cluster in pcopGFP lentivector (System Biosciences, Mountain View, CA) and pPACK packaging mixture. To produce lentiviral particles carrying doxycicline-inducible miR 17-92, a custom lentiviral vector was constructed, with constitutively expressed mCherry and M2rtTA (a reverse doxycycline transactivator), and a custom-made promoter with the doxycycline operator and a minimal CMV promoter, under which the miR 17-92, amplified by PCR from pLVX-eG2N-1792 (construct previously described), was cloned. Replication-incompetent lentiviral particles were then produced by calcium-phosphate transfection of HEK293T cells using the packaging plasmids pMD.2G (VSV-G) and pPAX2, as well as the doxycycline-induced miR 17-92 as the shuttle vector. The medium was replaced for fresh DMEM complete 6h after transfection, and 48h afterwards the medium was collected, cleared by low-speed centrifugation, filtered through 0.45 μm pore-size PVDF filters, and stored in aliquots at -80ºC. To construct the lentiviral vectors carrying 5 different shRNAs targeting p21, a forward and a reverse oligonucleotide for each shRNA was designed, in order to reconstitute the shRNAs through an hybridization PCR and introduce restriction sites at the 5' and 3' of the shRNA for posterior

cloning. The oligonucleotides are shown in **Table M&M2**. The shRNAs were cloned in the lentiviral vector pLVX-shRNA2 from Clontech, under the control of the U6 promoter and with zsGreen as a transduction control. Replication-incompetent lentiviral particles were produced by calcium-phosphate transfection of HEK293T cells using the packaging plasmids pMD.2G (VSV-G) and pPAX2, as well as either one of the shRNA plasmids as shuttle vectors. The medium was replaced for fresh DMEM complete 6h after transfection, and 48h afterwards the medium was collected, cleared by low-speed centrifugation, filtered through 0.45 μm pore-size PVDF filters, and stored in aliquots at -80ºC. Subsequently the viruses are titered by flow cytometry analysis of mCherry or GFP expression in 293T cells infected with increasing dilutions of virus. For infection of primary PDAC cells, a multiplicity of infection of 10 infectious units/ce was used and later sorted for mCherry, GFP or zsGreen. Tbx3 lentiviral vector and empty vector control were obtained from Origene.

*Table M&M2- shp21 sequences*

| shRNA | Sequence |
|---|---|
| p21.1 FW | gatccCCGGCTGATCTTCTCCAAGAGGAACTCGAGTTCCTCTTGGAGAAGATCAGCTTTTTGggtaccg |
| p21.1 RV | aattcggtaccCAAAAAGCTGATCTTCTCCAAGAGGAACTCGAGTTCCTCTTGGAGAAGATCAGCCGGg |
| p21.2 FW | gatccCCGGGCTGATCTTCTCCAAGAGGAACTCGAGTTCCTCTTGGAGAAGATCAGCTTTTTGggtaccg |
| p21.2 RV | aattcggtaccCAAAAAGCTGATCTTCTCCAAGAGGAACTCGAGTTCCTCTTGGAGAAGATCAGCCCGGg |
| p21.3 FW | gatccCCGGGACACCACTGGAGGGTGACTTCTCGAGAAGTCACCCTCCAGTGGTGTCTTTTTGggtaccg |
| p21.3 RV | aattcggtaccCAAAAAGACACCACTGGAGGGTGACTTCTCGAGAAGTCACCCTCCAGTGGTGTCCCGGg |
| p21.4 FW | gatccCCGGCCGCGACTGTGATGCGCTAATCTCGAGATTAGCGCATCACAGTCGCGGTTTTTGggtaccg |
| p21.4 RV | aattcggtaccCAAAAACCGCGACTGTGATGCGCTAATCTCGAGATTAGCGCATCACAGTCGCGGCCGGg |
| p21.5 FW | gatccCCGGGTCACTGTCTTGTACCCTTGTCTCGAGACAAGGGTACAAGACAGTGACTTTTTGggtaccg |
| p21.5 RV | aattcggtaccCAAAAAGTCACTGTCTTGTACCCTTGTCTCGAGACAAGGGTACAAGACAGTGACCCGGg |

## 8. STATISTICAL ANALYSES

Results for continuous variables are presented as means ± standard deviation unless stated otherwise and significance was determined using the Mann-Whitney test. All analyses were

performed using SPSS 17.0 (SPSS, Chicago, IL). If not otherwise stated, significance is given as $p<0.05$.

# RESULTS

# 1. FUNCTIONAL ANALYSIS OF PANCREATIC CANCER STEM CELL

It has shown that primary pancreatic CSCs can be enriched in vitro as anchorage-independent spherical colonies termed spheres (Hermann et al., 2007b). These spheres are composed of a subset of cells with stem cell-like properties including the ability to form secondary spheres as well as more differentiated progenies. Furthermore, the enrichment and isolation of pancreatic CSCs using surrogate cell surface markers, such as the pentaspan transmembrane glycoprotein CD133, also known as Prominin-1, has been reported to enriched in CSCs from freshly isolated patient-derived samples (Hermann et al., 2007b).

In the present study, we provide a more detailed characterization of spheres derived cells to functionally define the biological features of pancreatic CSCs. We used several human pancreatic adenocarcinoma xenografts or tumour-derived primary cell lines (A6L, 185, 354, 215 and 253) that have been previously described (Jones and Wagers, 2008, Rubio-Viqueira et al., 2006). Importantly, all cells for in vitro experiments were freshly isolated from early passage xenografts and cultured as adherent cells (monolayer) or anchorage-independent spheres at low passages (**Figure 1A**). Cells were phenotyped by flow cytometry for the expression of CSCs markers; as previously reported (Lonardo et al.), spheres are enriched in $CD133^{+}$ cells, as well as several other markers that have been associated with a CSC phenotype such as SDF-1 ligand CXCR4, and the differentiation marker stage-specific embryonic antigen-1 SSEA-1, as compared with adherent cells (**Figure 1B**).

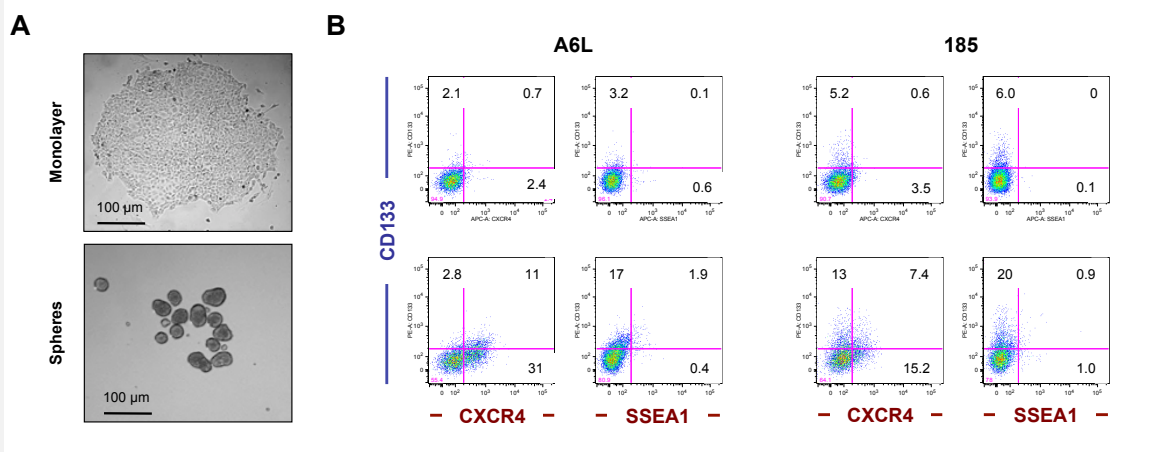



**Figure 1.** (**A**) Representative pictures of primary PDAC cells in monolayer and in spheres. (**B**) Flow cytometry of CD133+ CXCR4+ and CD133+ SSEA1+ cells is shown for primary PDAC A6L and 185 cells cultured as adherent cells or spheres.

## 1.1. Cancer stem cell related genes

In order to further characterize cancer stem cells in sphere culture, we determined the expression of pluripotency-associated genes (Nanog, Oct3/4, Stat3, Klf4, and Sox2) by real-time PCR that has been extensively described as the "core transcription network" in the regulation of pluripotency in human and mouse Embrionic Stem Cells (ESC) and Induced Pluripotent Stem Cells (iPS) (Maherali et al., 2008, Takahashi and Yamanaka, 2006, Takahashi et al., 2007, Kim et al., 2008). Expression of pluripotency-associated genes was significantly higher in first generation sphere culture (d7) versus 70% confluent monolayer culture (**Figure 2, left panel**). Intriguingly, the expression levels observed for pancreatic spheres were comparable to those of human ESCs (data not shown).

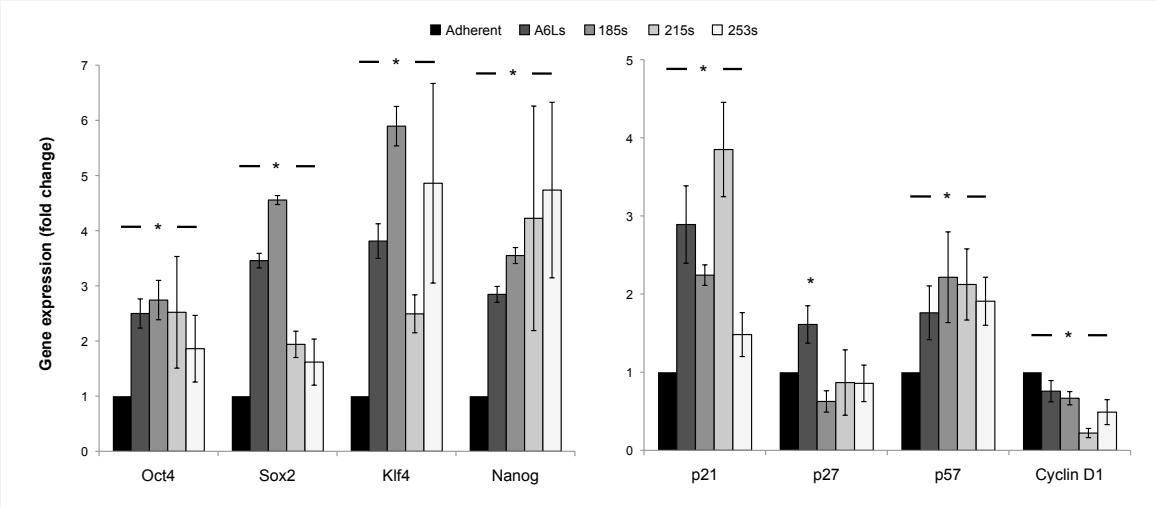



**Figure 2.** QPCR analysis of pluripotency-associated genes and cell cycle genes p21, p27, p57, Cyclin-D1 in adherent versus spheres derived cells. Data are normalized for ß-Actin expression.

## 1.2. Cell cycle analysis

To better characterize sphere-derived CSCs, we also analyzed the cell cycle profiles of sphere and adherent cultures, as previous work in other cancers suggest that cells with "stem-like" phenotypes bear distinct cell cycle profiles (Dembinski and Krauss, 2009, Chen et al., 2012, Li and Bhatia, 2011). We evaluated the expression of the cell cycle regulator genes p21, p27, p57, and Cyclin D1 at the mRNA level and we found significant over-expression of p21 and

p57 among several spheres derived cells, while Cyclin D1 is down regulated. p27 is significantly up-regulated only in A6L spheres derived cells (**Figure 2, right panel**).

Expression of protein levels was evaluated for Nanog, p21, p27, p57 and cyclinD1 by western blotting, validating the results observed for gene expression. Protein levels of p27 show a consistent up regulation in spheres derived cells (**Figure 3**), suggesting different post-translational modification or processing.

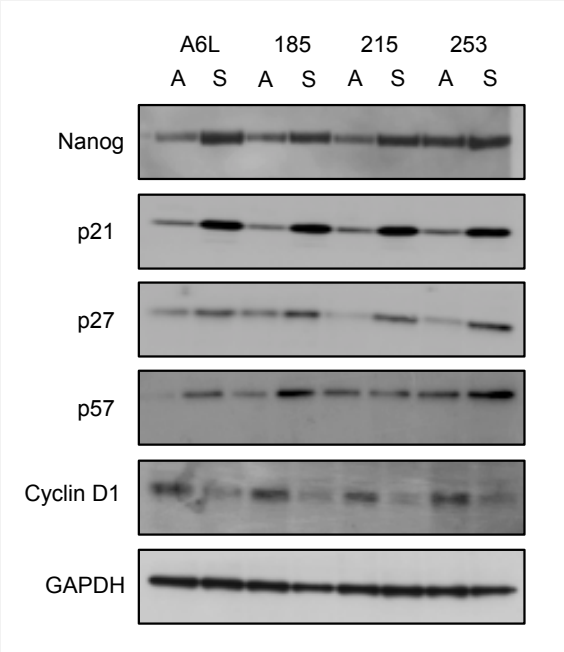



**Figure 3**. Western blot analysis of Nanog and cell cycle genes p21, p27, p57 and Cyclin-D1 in adherent cells compared with the spheres

We next analyzed the cell cycle profiles of sphere and adherent cultures, in order to functionally evaluate the change in gene and protein expression of the cell cycle proteins. Sphere-derived cells displayed a significant reduction of cells in S- and G2-M phase, which was accompanied by a notable enrichment for cells in G0 and G1 phases, respectively (**Figure 4**). Consistent differences were observed for primary cultures derived from other primary tumours tested (**data not show**).

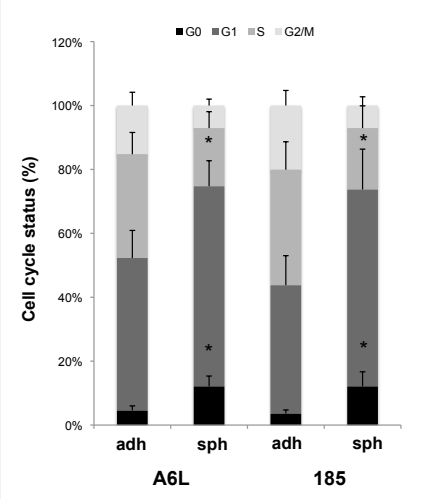



**Figure 4.** Cell cycle analysis using ki67 and Dapi

To further validate the presence of a slow-cycling subpopulation in our primary pancreatic cancer cells, we assessed quiescence by using the lipophilic fluorescent dye PKH26, which is a label retaining dye that stains the cell membrane. Those cells that are more quiescent (slow cycling) will retain the label longer compare to those with a fast cycling. In fact, PKH26 staining has recently been used to identify slow-cycling cancer stem cells in breast cancer (Pece et al., 2010) and melanoma (Roesch et al., 2010). The average doubling time of primary pancreatic cancer cells is ~36 hr. Only 1-3% of the cells retained the maximum amount of label after 4 weeks suggesting that those cells had not divided (**Figure 5**).

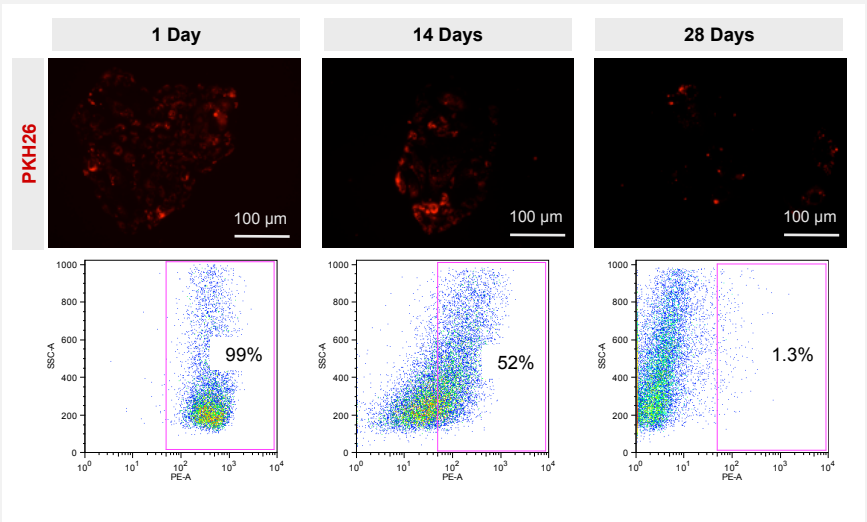



**Figure 5.** Representative pictures (**upper panel**) and flow cytometry analysis (**lower panel**) of PKH26 labeled cells after 1, 14 and 28 days in colture.

Flow cytometry analysis after 28 days in culture, reveals that PKH26 label-retaining cells expressed higher levels of surface CSC markers CD133, SSEA-1, and CXCR4 (**Figure 6A**). Moreover, when sorted, PKH26 label-retaining cells possess higher sphere formation capacity compared with negative cells (**Figure 6B**), suggesting that more quiescent cells possess CSC features in pancreatic cancer.

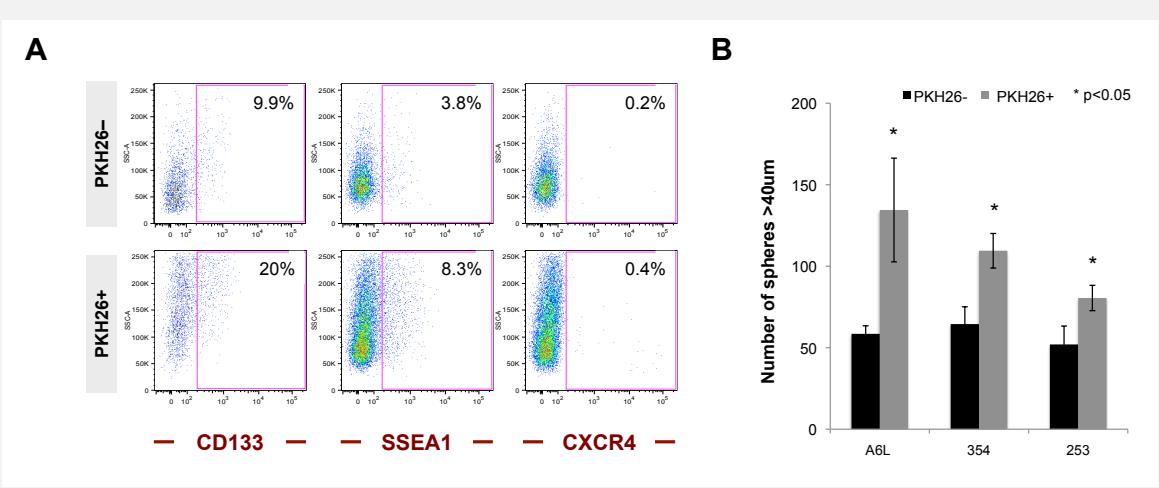



**Figure 6. (A)** Flow cytometry analysis for surface CSC markers CD133+, SSEA1+ and CXCR4+ in PKH26+ and PKH26- cells (**B**) Sphere formation capacity of PKH26+ and PKH26- cells in A6l, 354 and 253 cells. Each bar represents the mean sphere number ± SD (n=3; *P<0.05).

## 1.3. Chemoresistance

One of the clinical features of cancer stem cells is the ability to escape chemotherapy through several mechanisms and recapitulate the tumour heterogeneity, producing the relapse of the disease (Abdullah and Chow, 2013). We treated adherent cells and sphere derived cells with Gemcitabine (100ng/ml) during 48 hours and later we performed apoptosis analysis using AnnexinV assay. Interestingly we observed a massive increase of apoptotic cells in adherent cells, while spheres derived cells only show a modest response to the treatment (**Figure 7**).

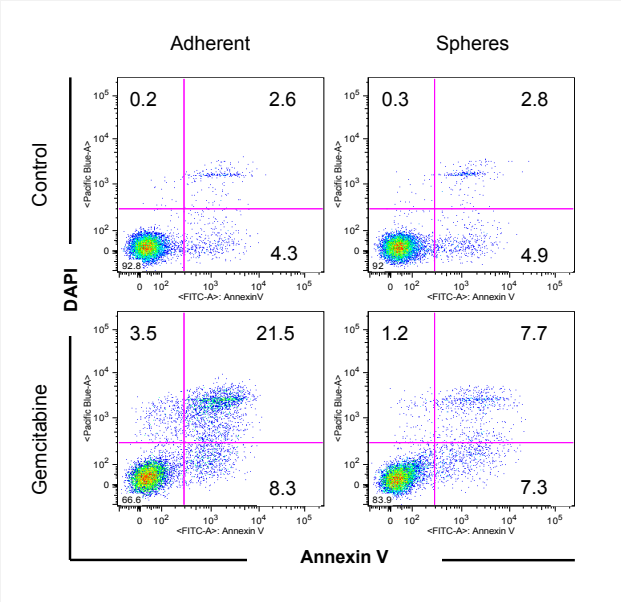



**Figure 7.** Flow cytometry analysis of apoptotic cells with AnnexinV and DAPI after treatment with Gemcitabine (100ng/ml) during 48 hours of adherent and sphere derived cells.

## 2. GEMCITABINE RESISTANT CELLS ARE ENRICHED FOR QUIESCENT CANCER STEM CELLS

The aforementioned experiments show that spheres derived cells are enriched in cancer stem cells and that cancer stem cells possess a slow-cycling population that can be responsible of the resistant to chemotherapy (Li and Bhatia, 2011). In order to validate our findings in a different setup, we decided to analyze, both *in vivo* and *in vitro,* the biology and the phenotype of chemoresistant cells after treatment with gemcitabine.

### 2.1. In vivo characterization of gemcitabine resistant cells

In accordance with the hypothesis that chemotherapy preferentially targets non-CSCs, we treated several human primary tissue xenografts in immunocompromised mice. Mice with tumours measuring ≥200 $mm^3$ (day 7) were then treated with Gemcitabine for 3 weeks (biweekly 125 mg/kg i.p.) (**Figure 8A**). Tumours were harvested, dissociated into single cells, and depleted for mouse hematopoietic cells and endothelial cells using MACS depletion for CD45 and CD146, respectively (**Figure 8B**) in order to have a more pure population for further analysis.

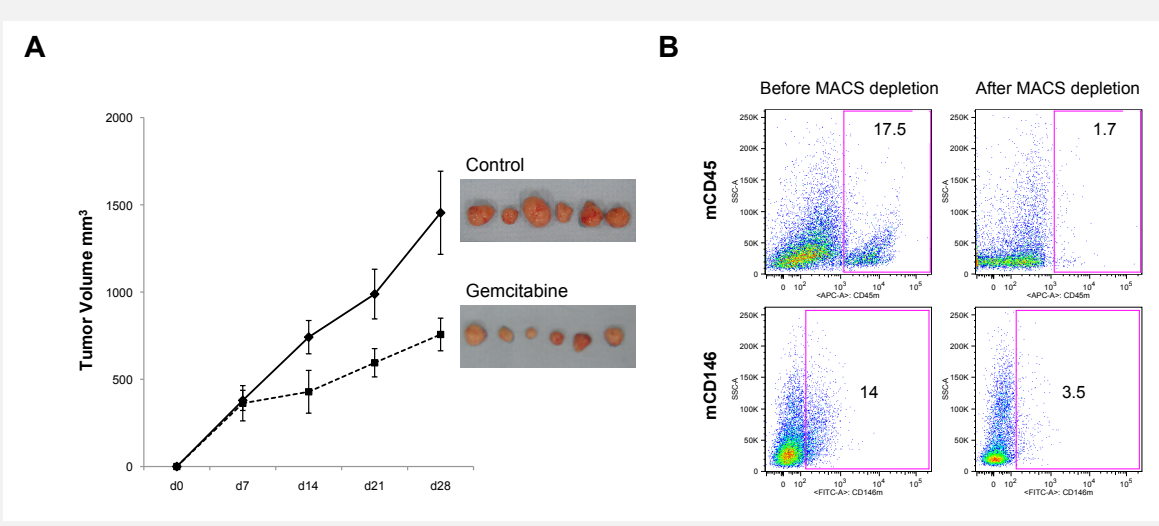



**Figure 8. (A)** Human primary xenografts were treated with vehicle or 125 mg/kg/ biweekly Gemcitabine (treatment started at day 7 and ended at day 28 of x-axis). Tumor diameters were measured using calipers, and volume in mm3 was calculated. **(B)** Flow cytometry analysis of gemcitabine treated tumors depleted by MACS for mouse CD45 and mouse CD146

Using the isolated and depleted cells from the tumours for flow cytometry analys, we observed a more prominent CSC subset in the Gemcitabine-resistant cell population that remained following treatment, defined by the increased expression of the surface markers CD133 and CXCR4 (**Figure 9a**). This apparent enrichment in CSCs was functionally validated at the level of sphere formation, where we observed markedly more sphere formation from Gemcitabine-treated tumour-derived cells compared to untreated controls (**Figure 9B**).

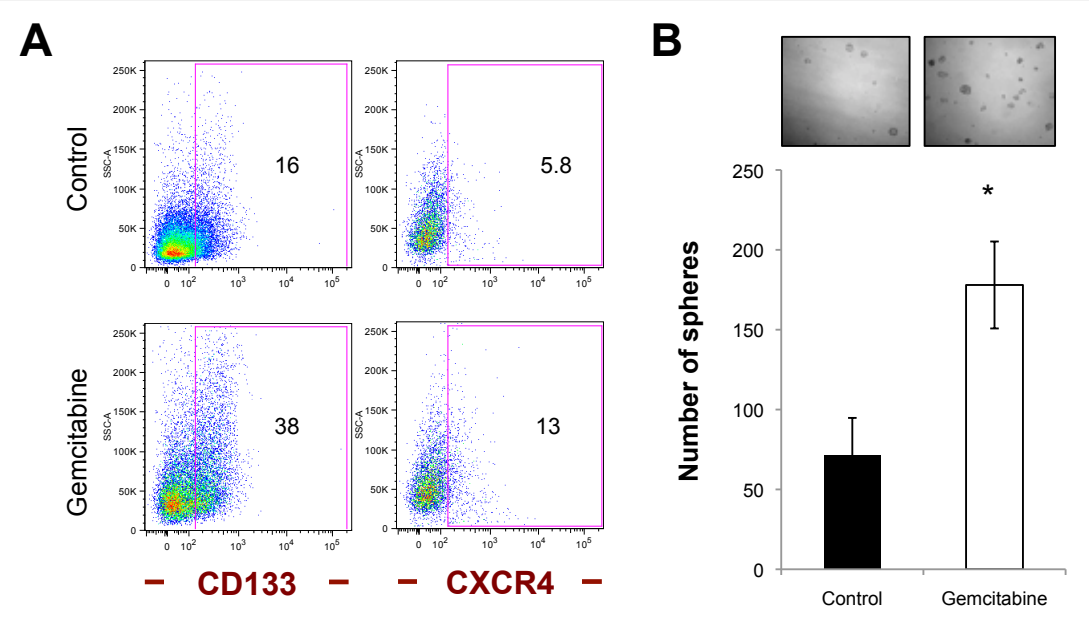



**Figure 9. (A)** Flow cytometry analysis for CD133 and CXCR4 after treatment with Gemcitabine. **(B)** Sphere formation capacity of Gemcitabine-resistant cells. Each bar represents the mean pancosphere number ± SD (n = 3; *P < 0.05). Original magnification, ×100.

To further characterize gemcitabine resistant cells, we performed gene expression analysis for stemness-related gene (Oct3/4, Sox2, Klf4, Nanog) and we observed that resistant cells possess "stem-like" phenotype due to overexpression of these gene compared with control cells (**Figure 10A**). Moreover, based on previous data showing the importance of Nodal/Activin signaling in the regulation of pancreatic cancer stem cells self renewal (Lonardo et al., 2011), we evaluate the expression of the genes involved in this pathway: Alk4, Nodal, Smad2, Smad4 in addiction to TgfbrII and Tgfb1. According to our hypothesis, we observed an increase in mRNA expression of the Nodal/Activin/TGF-β1 pathway members in Gemcitabine-treated tumours (**Figure 10B**).

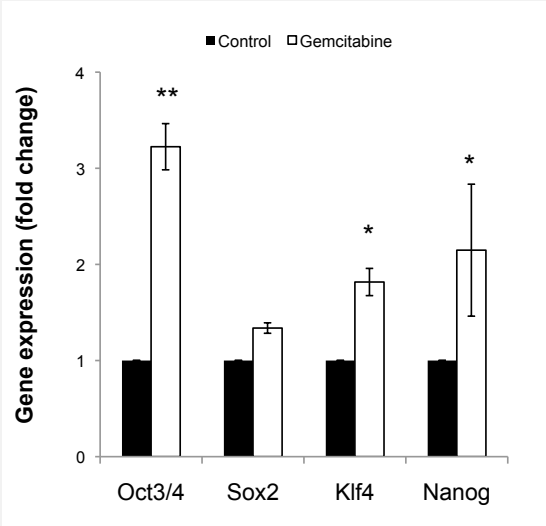



**Figure 10**. **(A)** Real-time PCR analysis for pluripotency associated genes (Oct3/4, Sox2, Klf4, Nanog) and **(B)** for genes involved in Nodal/Activin signaling (Alk4, Nodal, Smad2, Smad4, TgfbrII and Tgfb1)

## 2.2. *In vitro* characterization of gemcitabine resistant cells

To confirm these findings *in vitro*, we treated primary pancreatic cancer cell cultures 185 and A6L with Gemcitabine (100ng/ml) for 7 days, inducing >50% cell death. The CSC population, defined by CD133, CXCR4, and SSEA1 expression as assessed by flow cytometry, was increased in response to Gemcitabine (**Figure 11A**). Sphere formation capacity was also markedly increased by treatment with Gemcitabine in A6L cells and, albeit more modestly, in 185 cells (**Figure 11B**).

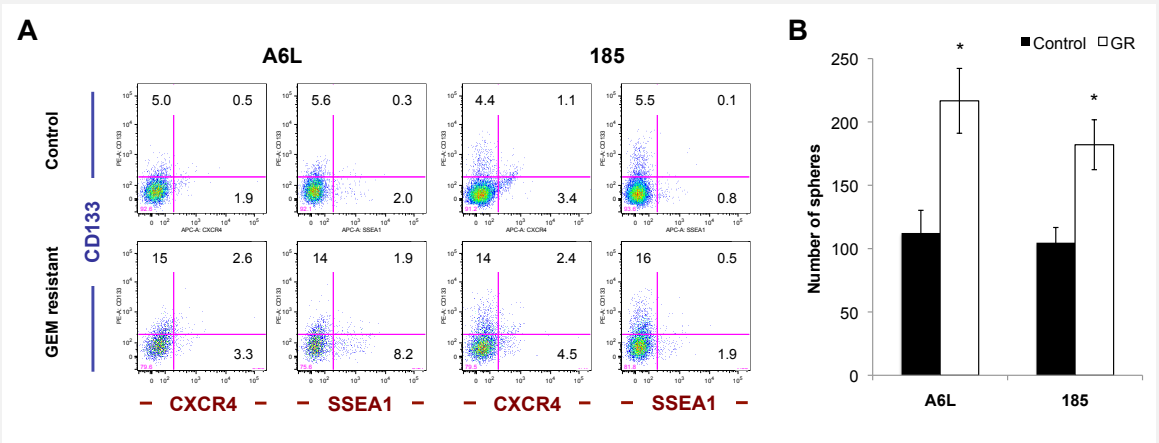



**Figure 11**. **(A)** Flow cytometry of CD133+CXCR4+ and CD133+SSEA1+ cells is shown for primary PDAC A6L and 185 cells cultured as adherent cells or treated with Gemcitabine (**B**) Sphere numbers of cell treated with Gemcitabine compared with control

Subsequent cell cycle analyses revealed an increase of cells in G0 and G1 phases accompanied by a decrease of cells in S-phase (**Figure 12A**) and an increase in PKH26 label-retaining cells (**Figure 12B**), suggesting that Gemcitabine-resistant cells reside in a more quiescent state. This hypothesis was confirmed by quantitative real-time PCR (qPCR) analysis of cell cycle genes revealing higher levels of p21 and p57 and down-regulation of Cyclin-D1 in Gemcitabine-resistant cells (**Figure 2C**).

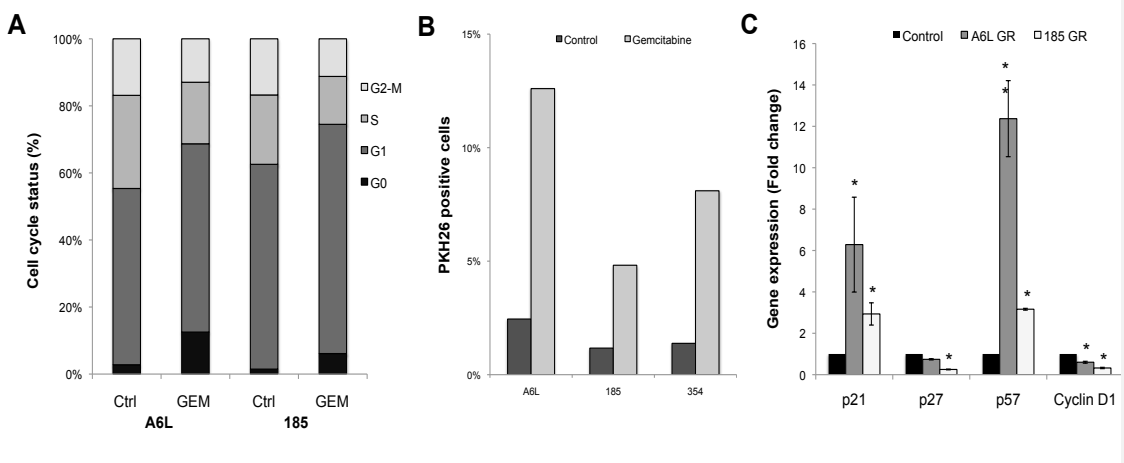



**Figure 12. (A)** Cell cycle analysis using ki67 and Dapi (**B**) Percentage of PKH26+ cells before and after gemcitabine treatment in A6L, 185 and 354 PDAC tumors. (**C**) QPCR analysis of cell cycle genes p21, p27, p57 and cyclin D1.

Moreover increased expression of stemness-related genes (Oct3/4, Klf4, Sox2, Nanog) was observed in Gemcitabine-resistant cells, a pronounced EMT transcriptional phenotype, with increased expression of Snail and Zeb1 and down-regulation of E-cadherin, was also observed. We also noted differential expression of cellular transporters implicated in drug resistance (de Wolf et al., 2008, Sharom, 2008), such as up-regulation of the ABC-transporters ABCC1 and ABCG2 and down-regulation of the Gemcitabine-specific transporters human concentrative nucleoside transporter (hCNT) and human equilibrative nucleoside transporter (hENT), both of which are necessary for Gemcitabine uptake and their expression levels are predictors of Gemcitabine response (**Figure 13A**) (Santini et al., 2010). We also observed an increase in mRNA expression of the Nodal/TGF-β1 pathway members ALK4, TGFBRII, SMAD2, SMAD4, and TBX3 (**Figure 13B**). Taken together, these data

demonstrate that culturing PDAC cells in the presence of Gemcitabine results in the enrichment of CSCs bearing multiple CSC features and properties.

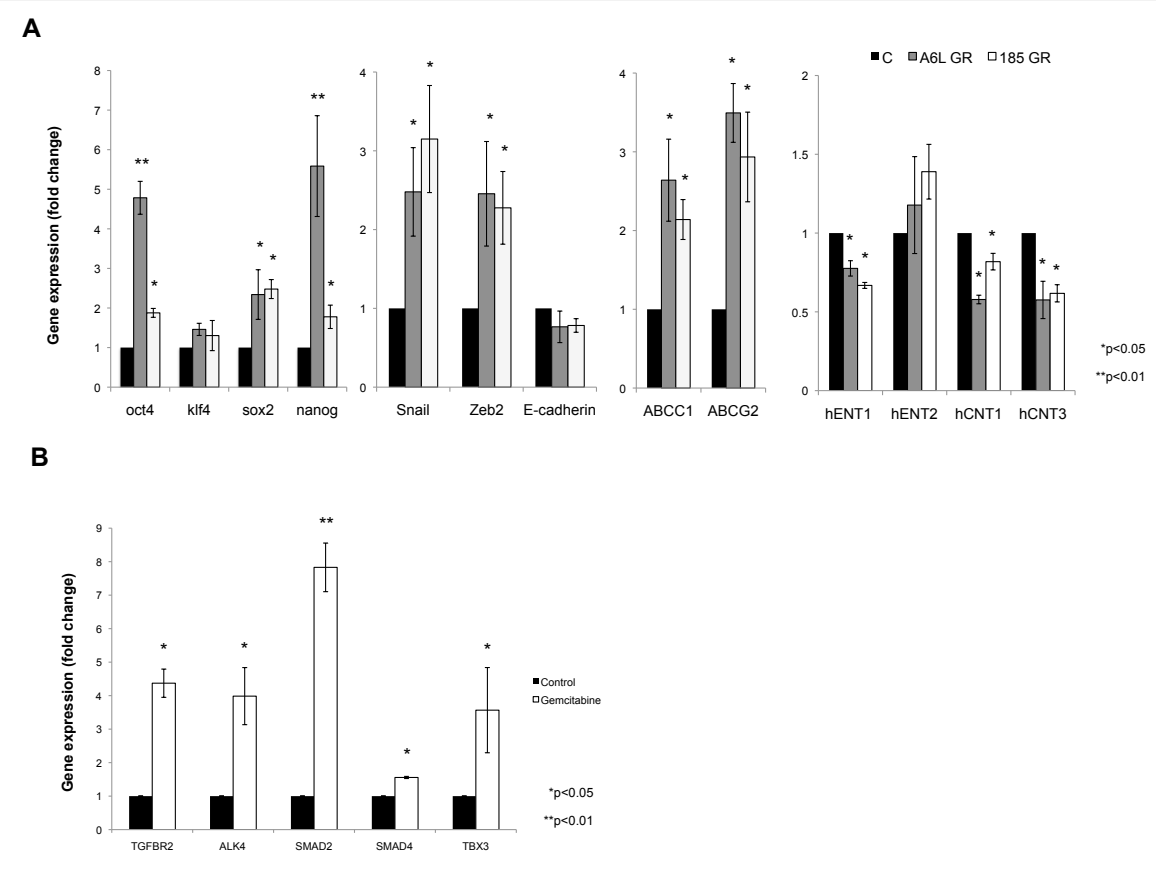



**Figure 13**. **(A)** QPCR analysis of pluripotency-associated genes (Oct4, Sox2, Klf4 and Nanog), EMT genes (Snail, ZEB2 and E-cadherin), ABC transporters (ABCC1 and ABCG2) and Gemcitabine transporters (hENT1, hENT2, hCNT1 and hCNT3). Data are normalized for ß-Actin expression. (**B**) QPCR analysis of TGFBR2, ALK4, SMAD2, SMAD4 and TBX3 in gemcitabine resistant cells compared with control.

## 3. THE MIR-17-92 CLUSTER IS DOWNREGULATE IN CANCER STEM CELLS

In order to identify miRNAs involved in the epigenetic regulation of self-renewal capacity and drug resistance of CSCs we compared miRNA expression profiles of 1) sphere-derived cells with adherent cells and 2) primary human xenografts implanted in immunocompromised mice treated with either vehicle or Gemcitabine for 3 weeks and depleted for mouse hematopoietic and endothelial cells. We performed a global normalization via quintile normalization for each set of sample pairs and subsequently ran a pairwise comparison via unpaired t-test and Benjamini Hochberg correction for false discovery rate. Subsequently we identified several miRNAs presenting statistically significant differences between control and Gemcitabine-resistant cells (**Figure 14A**).

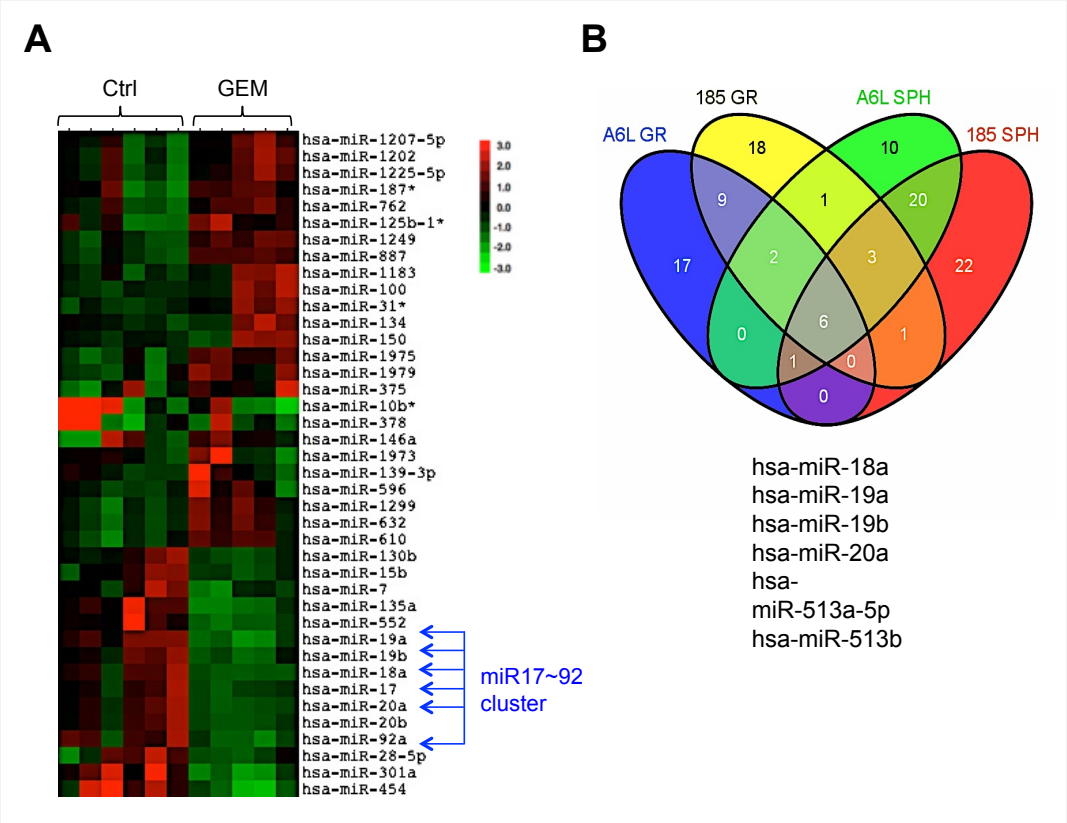



**Figure 14.** **(A)** Microarray analysis, represented as heat-maps, of differentially regulated miRNA in PDAC cells treated with Gemcitabine (red and green boxes indicate up- and down-regulation, respectively). **(B)** Venn diagram showing overlap between Gemcitabine-resistant and spheres downregulated miRNA.

As expected, we found commonly up-regulated miRNAs (miR-146a, miR-10b) along with commonly down-regulated miRNAs (miR-135a, miR-301, miR-7). Surprisingly, however, several members of the miR-17-92 family (miR-17, miR-18a, miR-19a, miR-19b, miR-20a*, miR-92a), which is commonly upregulated in bulk cancer tissue (Xiang and Wu, 2010), were also among the set of miRNAs downregulated in CSCs. To further validate our findings, we next compared the miRNA expression profile of Gemcitabine-resistant cells with the miRNA profiles obtained for sphere-derived cells versus adherent. Intriguingly, we again found consistent and statistically significant down-regulation of several members of miR-17-92 family (miR-18a, miR-19a, miR-19b and miR-20a) together with miR-513a-5p and miR-513b (**Figure 14B**). Importantly, we confirmed the down-regulation of several members of miR-17-92 family in an independent set of spheres derived cells and Gemcitabine-resistant cells by qPCR (**Figure 15A**) and reduction of miR-17-92 family members in PKH26-positive cells (**Figure 15B**). These data indicate that within highly proliferative bulk cancer cells, a subpopulation of CSCs resides in a slow-cycling state with low levels of miR-17-92 family members.

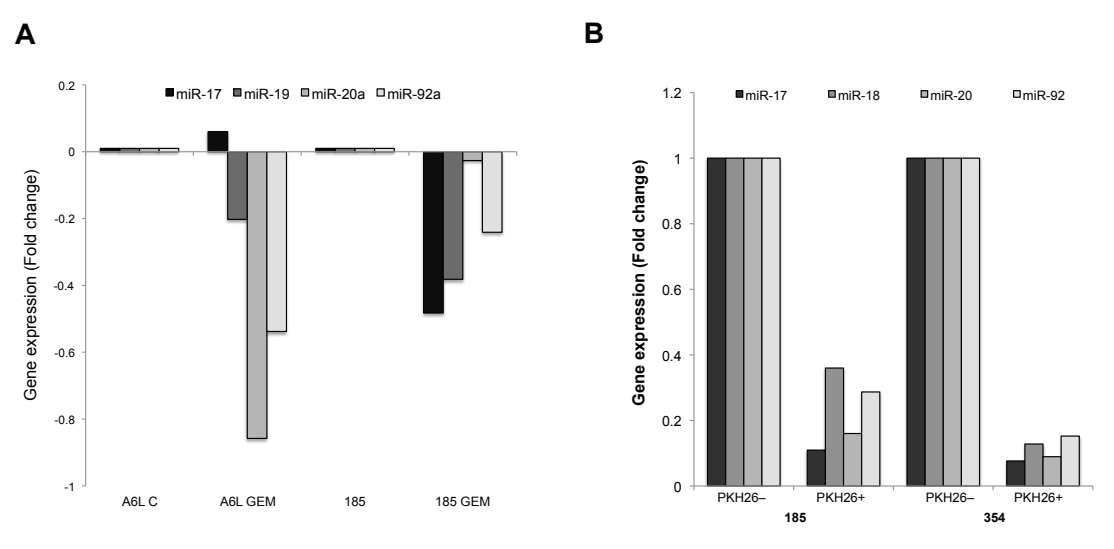



**Figure 15**. (**A**) QPCR analysis of the member of miR 17-92 family in Gemcitabine resistant cells and spheres. (**B**) QPCR analysis of the member of miR 17-92 family in PKH26+ and PKH26- cells from 185 and 354 PDAC tumors.

## 4. MIR-17-92 INHIBITION USING ANTAGOMIRS PROMOTES CANCER STEM CELL PHENOTYPE

In order to test the biological relevance of miR-17-92 in the context of pancreatic CSCs, we performed loss-of-function experiments by introducing an anti-sense inhibitor of miR-17-92 (termed miR-17-92 antagomir) conjugated with Cy3 into adherent pancreatic cancer cells that express high levels of this cluster and contain mostly differentiated cells, which are less tumourigenic compared to their less differentiated CSC counterparts (Lonardo et al., 2011). We transfected adherent cells derived from several primary pancreatic cancers that are more differentiated, with miR-17-92 antagomirs. We first assessed the expression of CSC surface markers CD133 by flow cytometry: we observed a significant increase in the expression of CD133 in several primary pancreatic cells (**Figure 16A**). Moreover, adherent cells trated with antagomir 17-92 show enhanced self renewal capacity as determined by sphere formation (**Figure 16B**) and overexpression of pluripotency-associated genes, ABC transporters and CD133 assessed by real time PCR (**Figure 16C**).

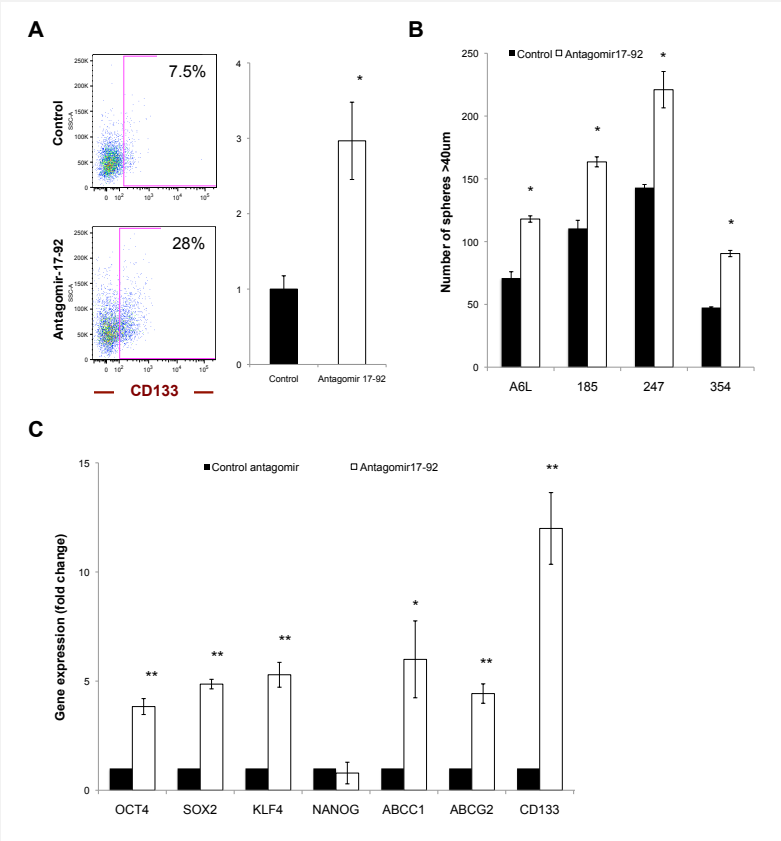



**Figure 16. (A)** Flow cytometry analysis for CD133 in cells treated with antagomirs. (**B**) Sphere numbers of cell treated with antagomir compared with control. (**C**) QPCR analysis of pluripotency-associated genes in cells treated with antagomirs. Data are normalized for ß-Actin.

Adherent cells possess a more proliferative and differentiated phenotype compared with the sphere-derived cells that are enriched in slow proliferating cells with CSC phenotype. Of note, adherent cells transfected with miR-17-92 antagomirs showed an increase in cells residing in G0 and G1 phase accompanied by a reduction of cells in S phase (**Figure 17A**). Protein analysis of antagomir treated cells, show a marked increase in p21 expression (**Figure 17B**) that correlate with the change in cell cycle analysis since has been already described as one of the key player in haematopoietic (Cheng et al., 2000) and cancer stem cell quiescence (O'Brien et al., 2012). These alteration in cell cycle and p21 expression, translated into an increased chemoresistance after Gemcitabine treatment, similar to that of purified CSCs (**Figure 17C**).

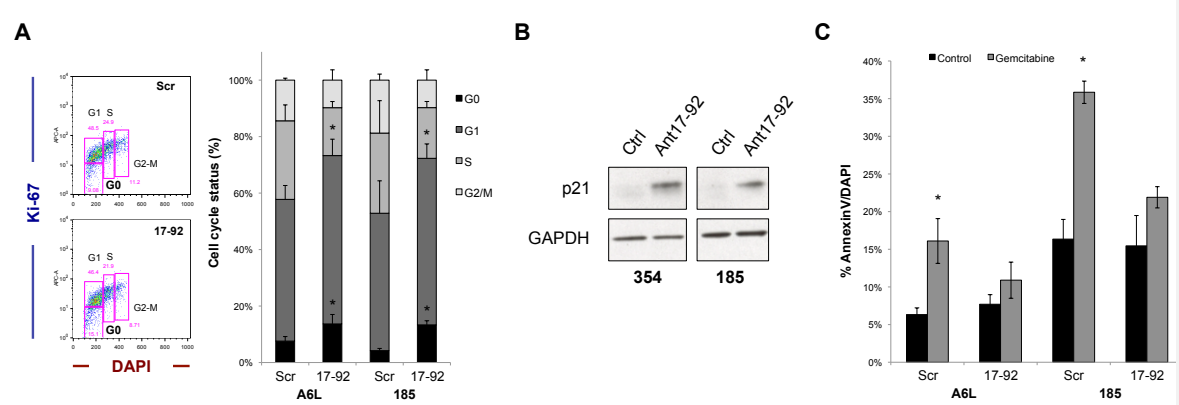



**Figure 17. (A)** Cell cycle analysis using ki67 and Dapi (**B**) Western blot analysis of cell cycle gene p21 in adherent cells treated with AntagomiR-17-92. **(C)** Chemoresistance by Annexin V/Dapi staining after treatment with Gemcitabine.

The most defining feature of CSCs is their ability to form tumours *in vivo*. We observed that adherent pancreatic cancer cells transfected with miR-17-92 antagomirs were markedly enhanced in their overall tumourigenicity (A6L: 4.2-fold [95% CI: 3.5-5.0] increase in CSC frequency; 185: 19.5-fold [16.2-23.4]), which did not only translate into higher tumour take rates, but also resulted in larger tumours compared to cells transfected with control antagomirs (**Figure 18 and data not shown**).

**Tumor take after two months**

(# tumors / # injections)

| | | $1x10^3$ | $1x10^2$ | Freq |
|---|---|---|---|---|
| **A6L** | control | 4/5 | 2/5 | 1/4522 |
| | ant17-92 | 5/5 | 3/5 | 1/1090 |
| **185** | control | 4/5 | 3/5 | 1/3596 |
| | ant17-92 | 5/5 | 5/5 | 1/1 |

**Figure 18.** In vivo tumorigenicity of A6L and 185 treated with Antagomir-17-92.

Taken together, these loss-of-function experiments in differentiated pancreatic cancer cells support the hypothesis that the miR-17-92 cluster negatively controls the tumourigenic capacity and chemoresistance of pancreatic CSCs.

## 5. OVEREXPRESSION OF MIR-17-92 REVERSES CANCER STEM CELL PHENOTYPE

Building upon our loss-of-function experiments using antagomirs, we next overexpressed miR-17-92 in pancreatic CSCs, where the expression of this cluster is significantly lower. For this purpose, we used a lentiviral construct (Lenti-miR) containing GFP and the common precursor of miR-17-92 (or scrambled control; Lenti-Ctrl) under the CMV promoter and an inducible system with doxicyclin containing mCHERRY. We first confirm up-regulation of miR-17-92 family members in PDAC spheres infected with Lenti-miR (**Figure 19**) and then we performed several experiments to evaluate the changes in CSCs phenotype.

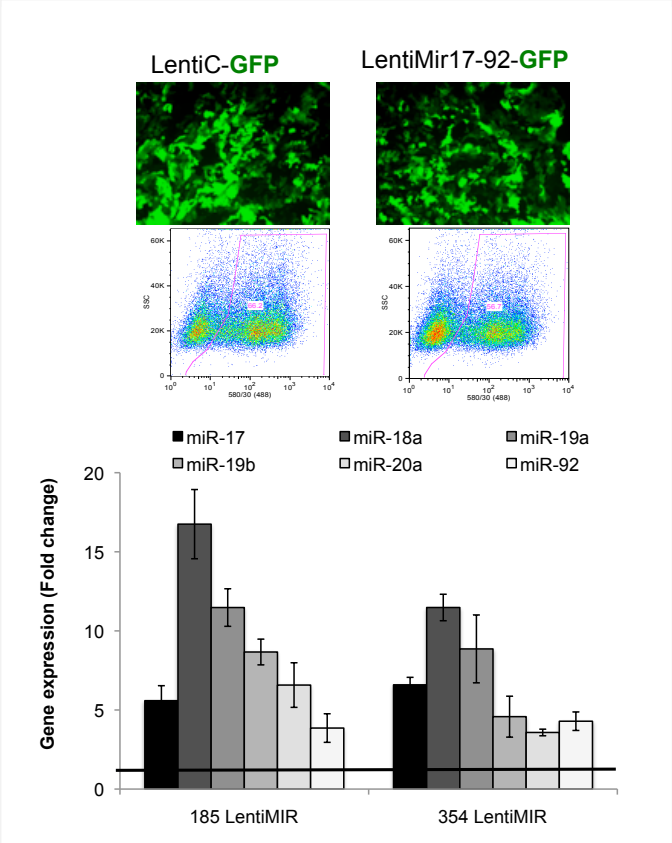



**Figure 19.** Lentiviral overexpression of miR 17-92 in spheres derived cells: representative images of GFP expression in spheres derived cells post infection and sorting strategy (**upper panel**) and qPCR of miRNA after sortig for GFP (**lower panel**).

## 5.1. Cancer stem cell phenotype

Using flow cytometry analyses we assess the expression of the CSC surface markers CD133 and SSEA-1, and observed that both markers were significantly decreased in Lenti-miR cells (**Figure 20A**), suggesting a possible transition of CSCs to a more differentiated phenotype. Along these lines, we also observed reduction in the expression of pluripotency associated genes (Oct4, Sox2, Klf4 and Nanog) (**Figure 20B**) and diminished sphere formation capacity across primary cells derived from several tumours (**Figure 20C**) following overexpression of the miR-17-92 cluster.

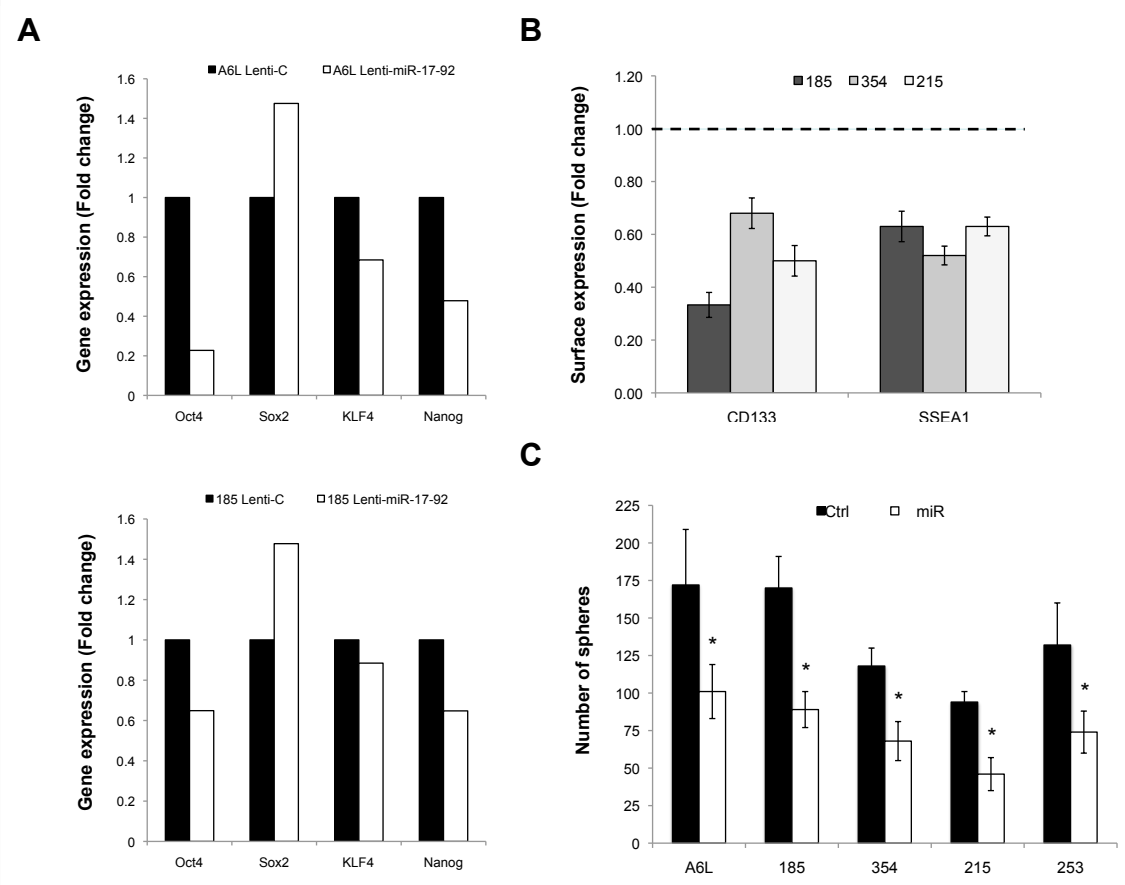



**Figure 20. (A)** QPCR analysis of pluripotency-associated genes (Oct4, Sox2, Klf4 and Nanog) in cells overexpressing miR-17-92 compared with the control. **(B)** Fold change in surface expression of CD133 and SSEA1 in cells overexpressing miR-17-92 compared with control. (**C**) Sphere numbers of cell overexpressing miR-17-92 compared with the control.

Moreover, cell cycle analysis revealed a decrease in cells residing in G0- and G1-phase and an increased fraction of cells in S-phase upon overexpression of miR-17-92 indicating a less quiescent phenotype (**Figure 21A**). Importantly, we did not observe alterations in the level of β-galactosidase staining indicating that there are no changes in terms of senescence (**Figure 21B**). This hypothesis was functionally confirmed by PKH26 labeling of cells overexpressing miR17-92 that show a rather reduced quiescence compared with control cells (**Figure 21C**).

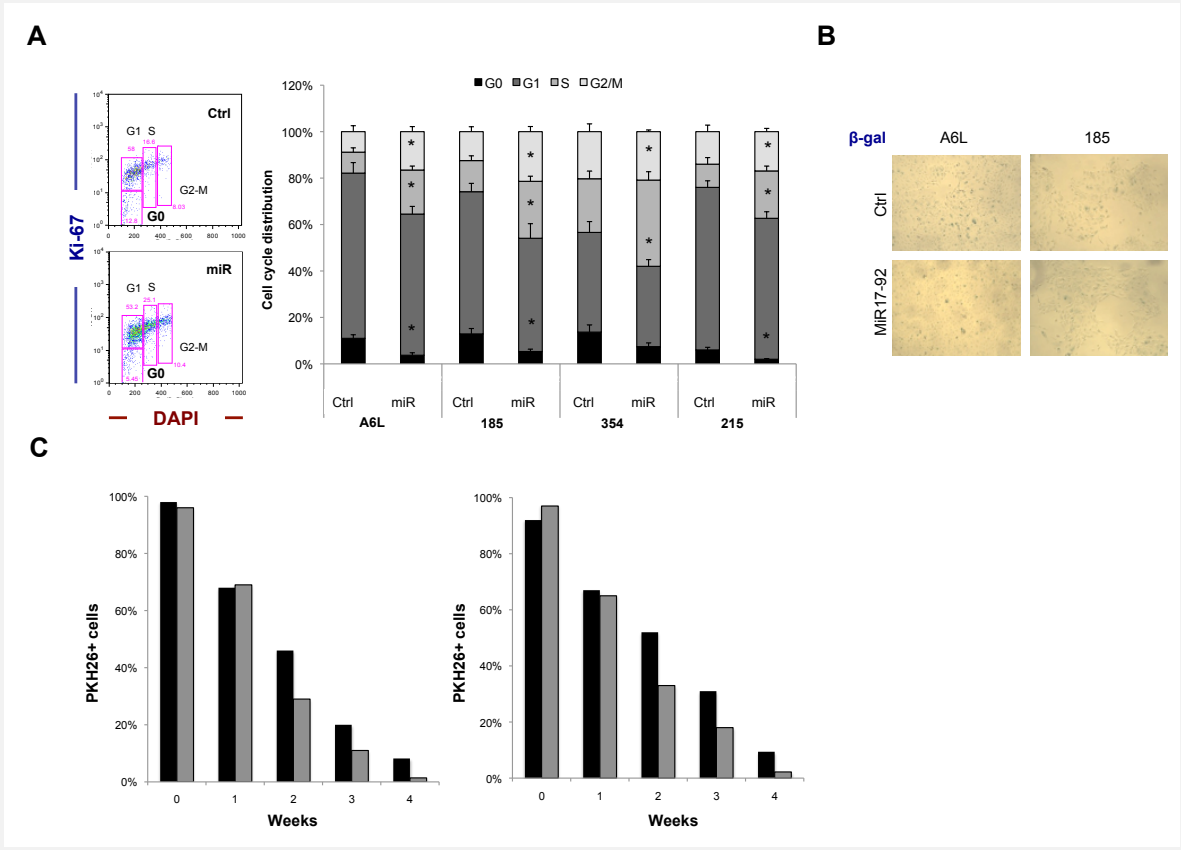



**Figure 21.** **(A)** Cell cycle analysis using ki67 and Dapi **(B)** Representative images showing β-galactosidase staining in spheres derived cells overexpressing miR-17-92 compared with control. (C) Percentage of PKH26+ cells during 4 weeks in spheres derived cells overexpressing miR-17-92 compared with control

These changes in terms of CSC content and cell cycle are translated into marked increase in chemosensitivity after 7 days treatment with Gemcitabine (**Figure 22**).

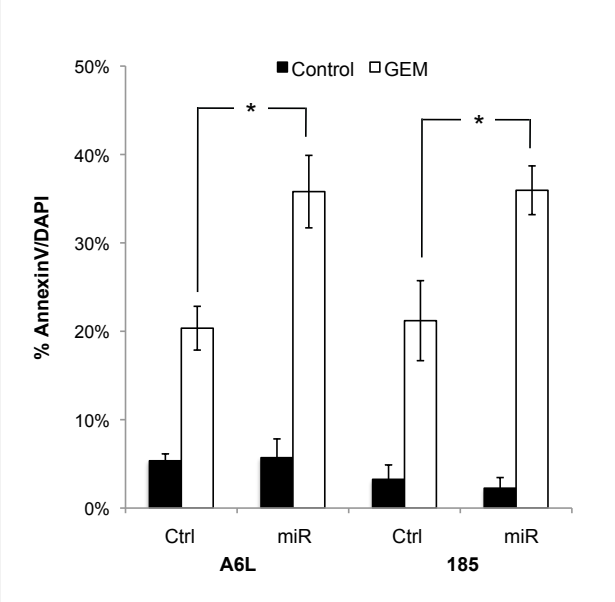



**Figure 22.** Chemoresistance by Annexin V/Dapi staining after 7 days treatment with Gemcitabine.

## 5.2. *In vitro* and *in vivo* migration capacity

Within the tumour exist a subpopulation of migrating cancer stem cells (CSCs) represents the exclusive source for metastasis in a model of pancreatic cancer (Hermann et al., 2007b, Hermann et al., 2008). Based on previous studies, Nodal/TGF-β1 signaling plays a pivotal role in the migration and invasion of pancreatic CSCs (Lonardo et al., 2011, Gaspar et al., 2007, Teraoka et al., 2001), To further investigate the migrating and invasive capacity of the primary pancreatic cancer cells after overexpression of miR-17-92, we used several chemoattractant based on the previous studies linking them into a more CSC associated phenotype: TGF-β1 Nodal and Activin. Using wound healing assay to measure the migrating capacity, we observed that control cells responded robustly to TGF-β1 and Nodal, respectively, while cells over-expressing miR-17-92 had completely lost their responsiveness (**Figure 23A**). As expected the small molecule inhibitor of Alk4 and TGFBR2 reversed the capacity of control cells to migrate in the presence of TGF-β1 or Nodal, the effect was more pronounced in cells over-expressing miR-17-92, indicating an impairment of the Nodal/TGF-β1 pathway even prior to treatment with SB431502. To confirm our results in a

different experimental setting, we used Boyden chamber invasion assays and observed that the invasion capacity of cells over-expressing the miR-17-92 cluster was also strongly reduced in the presence of Nodal, Activin and TGF-β1 (**Figure 23B**).

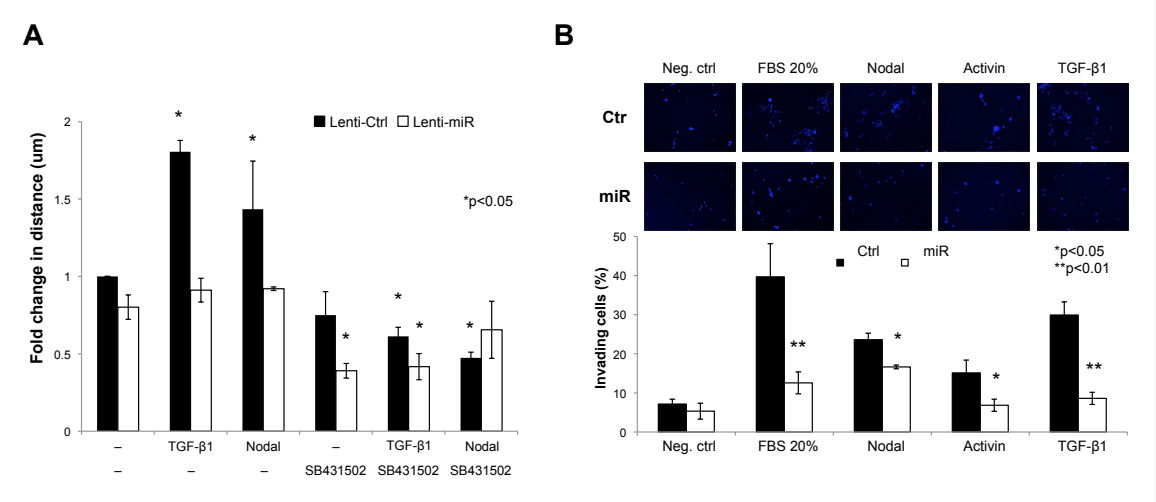



**Figure 23. (A)** Quantification of migration using wound-healing assay with stimulation of Nodal, TGF-ß1 and inhibition with SB 431502. **(B)** Representative images of invaded cells **(upper panel).** Percentage of invading cells through Matrigel™ following stimulation with Nodal, Activin or TGF-ß1 **(lower panel).**

To further validate these findings *in vivo*, we injected sphere-derived Lenti-Ctrl and Lenti-miR infected cells, respectively, into the spleen of NSG mice to assess liver dissemination and subsequent metastasis. Ten weeks post-injection, we observed reduced cell dissemination to the liver (i.e. micro-metastases) in mice injected with cells overexpressing miR-17-92, as determined by immunohistochemistry of the livers and qPCR for mCHERRY and hGAPDH (**Figure 24A).** Our findings were corroborated by *ex vivo* whole tissue immunohistochemistry demonstrating the presence of cells positive for human-specific ALU and huCK19 (**Figure 24B**).

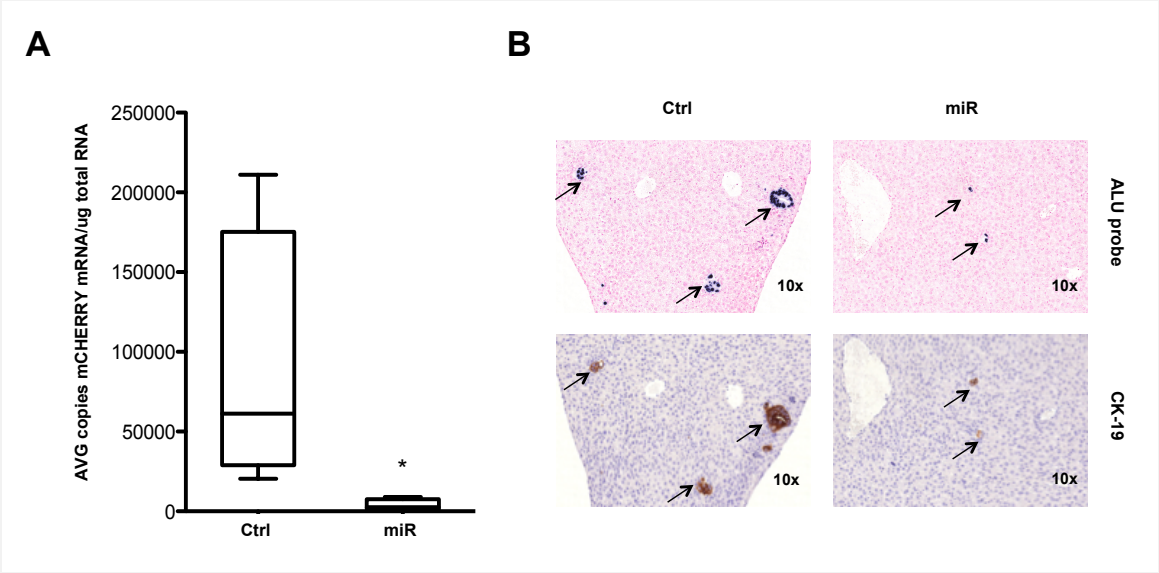



**Figure 24**. **(A)** QPCR quantification of mCHERRY copies for µg of total RNA extracted from fresh livers after intra-splenic injection of $5*10^4$ cells overexpressing miR-17-92 and control into NSG mice. **(B)** Representative immunohistochemistry images of pancreatic cancer cell human micrometastasis in mouse livers 10 weeks after intrasplenic injection. Arrows indicate cells stained positive for human-specific ALU probe and cytokeratin19.

## 5.3. *In vivo* tumourigenicity

Interestingly, despite the higher proliferation rate of cells transduced with miR-17-92, these cells produced significantly fewer tumours in a dose-dependent manner compared to cells transduced with a miR-Control construct (**Figure 25A**). Based on our *in vitro* results demonstrating that miR-17-92 expression reverses CSC quiescence, we next asked whether miR-17-92 overexpression specifically affects the long-term repopulation capacity of CSCs as assessed by serial transplantation of the tumours. During the first *in vivo* passage, we observed a higher proliferation rate for miR-17-92 overexpressing cells compared to the control tumours (**Figure 25B**), which closely mimicked the proliferation pattern of these cells *in vitro*. In contrast, however, during the subsequent two *in vivo* passages, miR-17-92 overexpressing cells gradually lost their potential to expand and eventually exhausted.

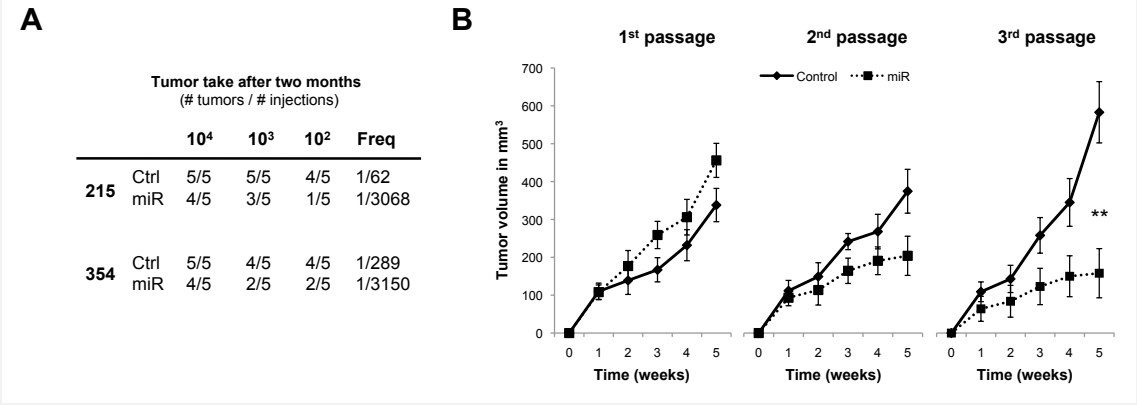



**Figure 25**. **(A)** *In vivo* tumorigenicity of cells overexpressing miR-17-92 compared with control cells and (**B**) *in vivo* serial transplantation.

These results were further corroborated by sphere formation assays using cells harvested at the end of each of the three *in vivo* passages. Consistently, we found that the self-renewal capacity of miR-17-92 overexpressing cells had decreased over time *in vivo* (**Figure 26A**).
Cell cycle analysis validated the initial increase in proliferation, which eventually culminated in their exhaustion (**Figure 26B**). Since cell-cycle restriction has been suggested as a mechanism to limit DNA damage and subsequent stem cell loss (Viale et al., 2009), we next assessed the level of DNA damage. For this purpose, the extend of phosphorylated histone H2A variant H2AX (δH2AX), which forms foci at sites of DNA double-strand breaks, was determined by confocal microscopy. In line with our cell cycle and *in vivo* exhaustion data, we found that cells over-expressing miR-17-92 showed more frequently gH2AX foci as compared to Lenti-Ctrl cells; a difference that further increased upon treatment with Gemcitabine (**Figure 26C**).

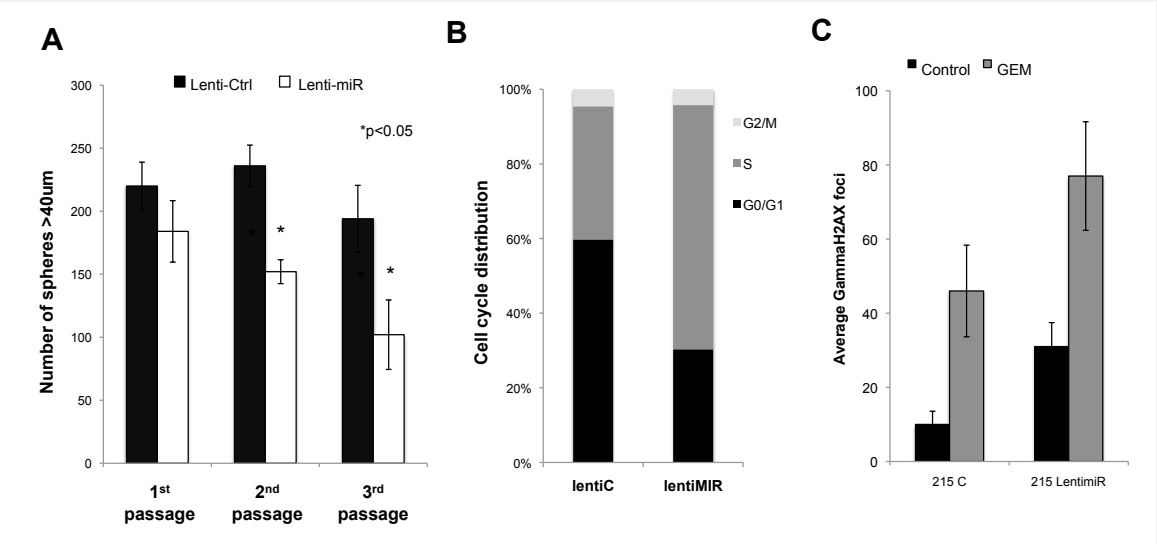



**Figure 26**. **(A)** Numbers of spheres during in vivo passages. (**B**) Cell cycle analysis using ki67 and Dapi of freshly isolated cells after in vivo serial transplantation. **(C)** Quantification of gH2AX staining (n = 100 cells counted) is shown ± SD (*P < 0.05).

Together, these results suggest that over-expression of miR-17-92 in CSCs results in loss of cell-cycle restriction/checkpoints by downregulation of p21 leading to an accumulation of DNA damage and eventually this mitotic chaos translates into CSCs exhaustion (Viale et al., 2009, O'Brien et al., 2012).

## 6. MIR-17-92 TARGETS NODAL/TGFβ SIGNALING

The miRNAs exert their regulatory effects by binding to complementary sites within the 3' untranslated regions (UTRs) of their mRNA targets. The formation of the double-strand RNA, resulting from the binding of the miRNA, leads to translational repression (Bartel, 2004). We took advantages of one of the available miRNA targets databases, TargetScan (http://www.targetscan.org), to search for potential targets of miR-17-92. We identified conserved binding sites for several members of miR-17-92 cluster in the 3'-UTR region of the Activin-like 4 (*ALK4),* TGF-β Receptor-2 *(TGFBR2), SMAD2,* and *SMAD4* genes, a core group of regulatory genes known to govern the stemness and/or metastasis of pancreatic CSCs (Lonardo et al., 2011), p21 and p57, main regulators of quiescence (Matsumoto et al., 2011, Kippin et al., 2005, Cheng et al., 2000) and TBX3, which has been implicated in the regulation of self-renewal of embryonic stem cells (Lu et al., 2011) and CSCs (Fillmore et al., 2010) (**Figure 27A and Figure 27B**).

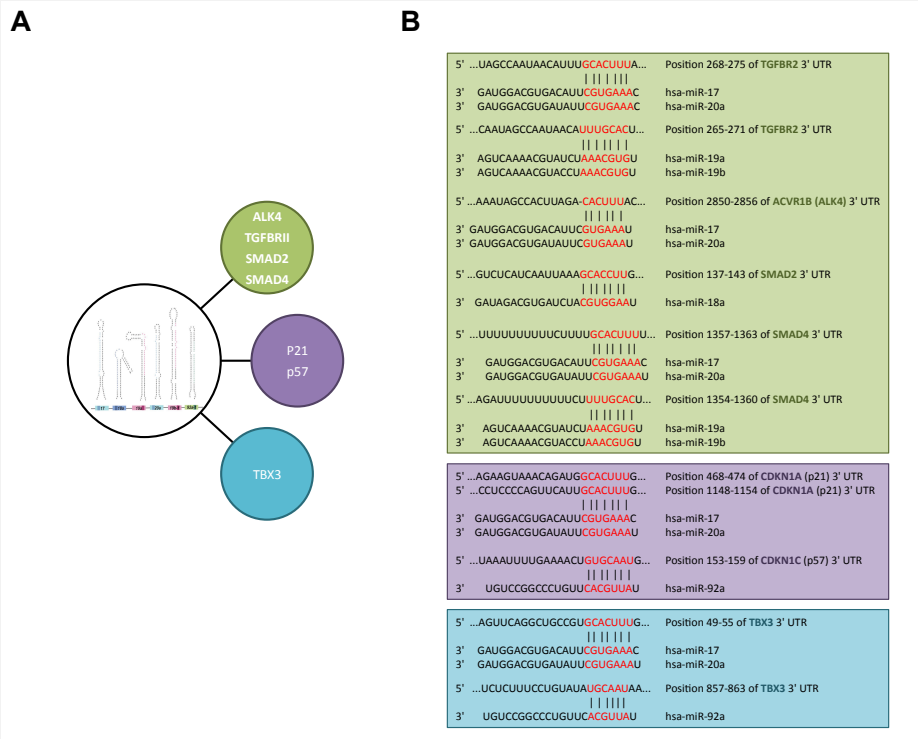



**Figure 27. (A)** Grafic representation of predicted targets of miR-17-92 cluster. (**B**) Sequence alignment of miR-17-92 family seed sequence in *TGFBR2* 3′ UTR, *ALK4* 3′ UTR, *SMAD2* 3′ UTR, *SMAD4* 3′ UTR, *p21* 3′ UTR, *p57* 3′ UTR and *TBX3* 3′ UTR.

To validate these genes as direct functional targets of miR-17-92 in primary PDAC cultures, we analysed a panel of primary pancreatic cancer cells expressing doxycycline-inducible Lenti-miR-17-92 or miR-control. After 96h treatment with doxycycline, we performed flow cytometry analysis for ALK4 cell surface expression and we observed a decrease in several primary pancreatic cancer cells upon overexpression of miR-17-92 (**Figure 28A**). Moreover, western blot analysis for p21, p57, pSMAD2 and Tbx3 shows a marked reduction of the protein levels after stimulation with doxycyclin and increase expression of miR-17-92 (**Figure 28B**).

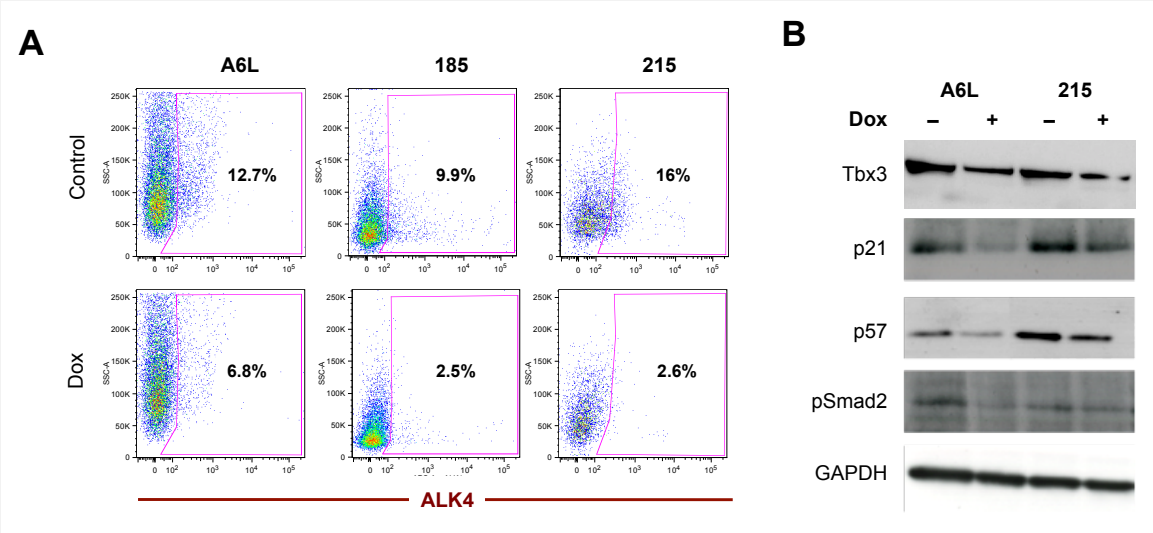



**Figure 28. (A)** Flow cytometry analysis for ALK4 before and after treatment with Doxicyclin 96h. (**B**) Western blot analysis of cell cycle gene p21, p57, pSMAD2 and TBX3. Normalization using GAPDH

We further validated the predicted targets analyzing the RNA expression by qPCR; for all target genes, we observed a marked reduction in their expression in CSCs over-expressing miR-17-92. (**Figure 29A**). We also validate the regulation of the predicted targets by single members of miR-17-92 cluster by qPCR using antagomirs, which reflect the predictions obtained for single family members using Targetscan (**Figure 29B**).

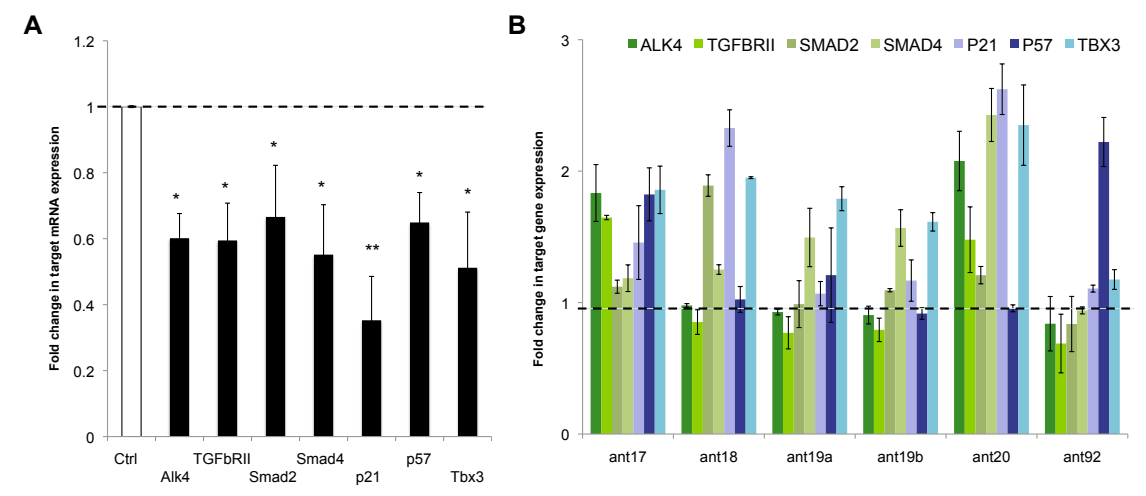



**Figure 29**. **(A)** QPCR analysis of miR-17-92 target genes after 72h treatment with Doxicyclin. Data are normalized for ß-Actin expression. (**B**) QPCR analysis of miR-17-92 target genes after 24h treatment with single antagomirs against the six members of the cluster.

To examine whether miR-17-92 members were able to interact with the 3′ UTR of Alk4 and Tbx3, we conducted a luciferase reporter assays. The complete 3′ UTR of Alk4 or Tbx3 gene was cloned into the GLuc Dual-luciferase reporter vector. The cells were co-transfected with GLuc vector containing the 3′ UTR of Alk4 or Tbx3 and miR-17-92 mimics: the results showed significantly lower expression of the luciferase compared with the cells transfected with the same reporter vector and control microRNA mimics (NC) (**Figure 30**).

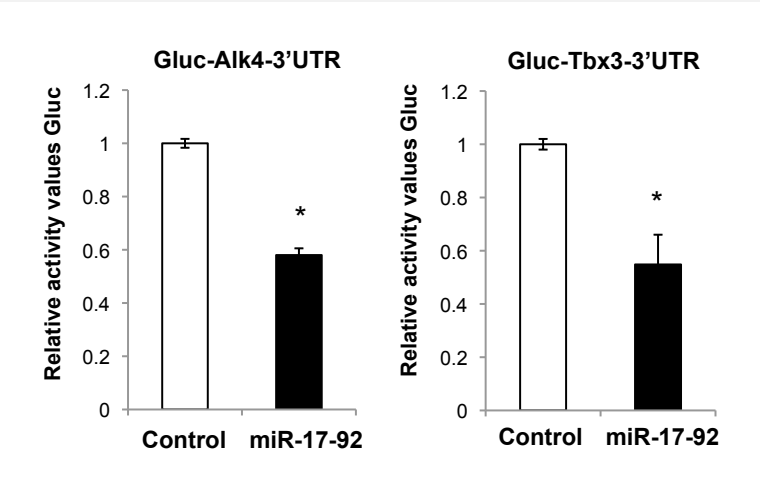



**Figure 30**. The complete 3′ UTR of Alk4 and Tbx3 genes were cloned into the GLuc Dual-luciferase reporter vector and co-transfected with miR-17-92 mimics and control miR mimics, respectively. The Gaussia luciferase activity was measured and normalized to Renilla luciferase activity (mean±s.d.; n = 3; *, P<0.05).

To functionally validate SMAD4 as direct target of miR-17-92, we used a pCAGA12-luciferase SMAD4 reporter in primary spheres derived cells overexpressing miR-17-92. After stimulation with Tgf-β1, Nodal and Activin we were able to observe an increase in the luciferase signal in control cells, while the effect is significantly reduced in cells overexpressing miR-17-92 meaning that SMAD4 is strongly inhibited by miR-17-92 (**Figure 31A**). Subsequently, we used the same cells stimulated with Tgf-β1, Nodal and Activin to evaluate the ability to induce the expression of p21, Tbx3 and pSMAD2. In control cells we observed an increase of p21, Tbx3 and pSMAD2 both at RNA and protein level, while in cells overexpressing miR-17-92, this effect is abrogated (**Figure 31B**).

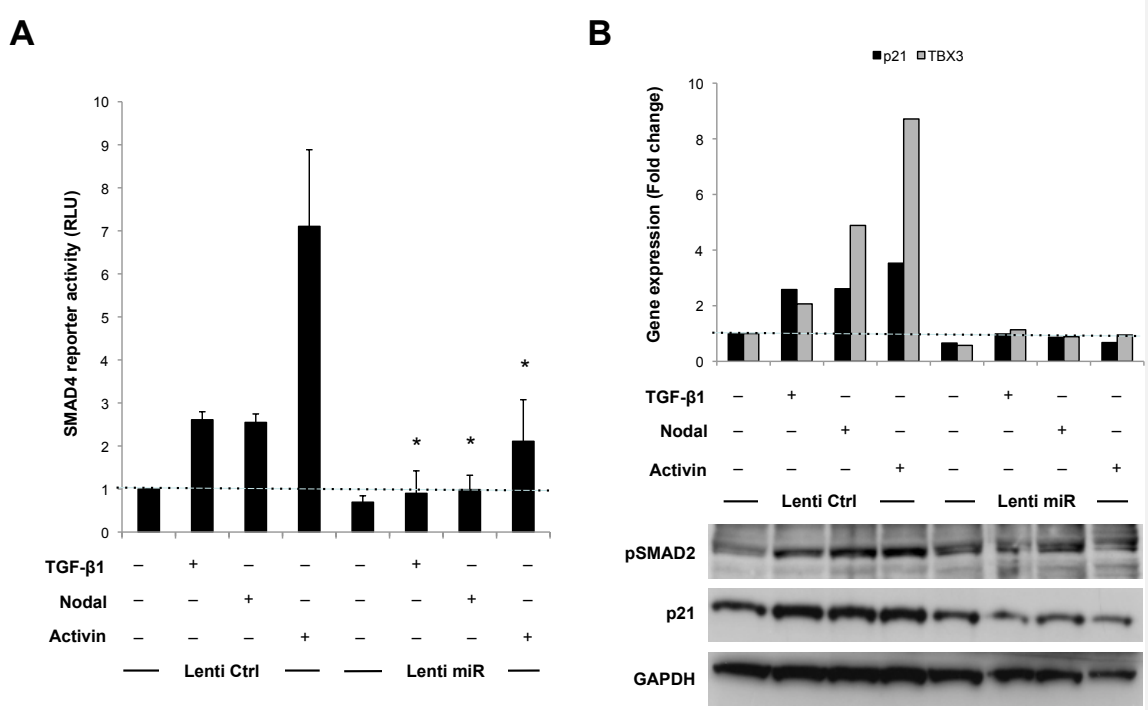



**Figure 31. (A)** Luciferase activity of pCAGA12-luc SMAD4 reporter after stimulation with Tgf-β1, Nodal and Activin in cell overexpressing miR-17-92 versus control cells. **(B)** QPCR for p21 and Tbx3 (**upper panel**) and western blot analysis for pSMAD2, p21 and GAPDH (**lower panel**) after stimulation with Tgf-β1, Nodal and Activin in cell overexpressing miR-17-92 versus control cells.

# 7. IDENTIFICATION OF MASTER REGULATORS OF SELF-RENEWAL AND CHEMORESISTANCE OF PANCREATIC CANCER STEM CELLS

In order to functionally validate the importance of miR-17-92 target genes in the regulation of CSC self-renewal and chemoresistance, we decided to perform knockdown experiments using shRNAs against target protein identified previously. Has been already shown, in a previous work, that genetic targeting using shRNA against Alk4, Nodal and SMAD4 reduced sphere formation capacity and CD133 expression and abrogates in vivo tumourigenicity of primary pancreatic cancer cells (Lonardo et al., 2011). We decided to knockdown p21 and Tbx3 in pancreatic cancer stem cells to determine the involvement in self-renewal capacity, invasion and chemoresistance.

## 7.1. p21 regulates quiescence and chemoresistance

Emerging evidences suggest that p21 plays a pivotal role in maintaining stem cell quiescence where its absence induces a rapid exhaustion of haematopoietic stem cells (HSCs) (Cheng et al., 2000). More importantly p21-induced cell cycle arrest is required for self-renewal because prevent the accumulation of excessive DNA damage and functional exhaustion of rapidly dividing leukaemia cells (Viale et al., 2009) and colon cancer stem cells (O'Brien et al., 2012). We asked whether p21 could possess the same properties in the contest of pancreatic cancer stem cells. Analysis of spheres derived cells enriched for chemoresistant CSCs shows a marked increase of p21 at protein and RNA levels compared with the more differentiated a proliferative adherent cells (**Figure 2 and Figure 3**). We used a short-hairpin RNA (shRNA) targeting p21 (sh-p21) to specifically suppress the expression of p21 in a panel of primary pancreatic spheres derived cells with a different degree of inhibition assessed by densitometric quantification (**Figure 32A**). Knockdown of p21 also inhibited sphere formation capacity during serial passages in several primary pancreatic cancer cells (**Figure 32B**).

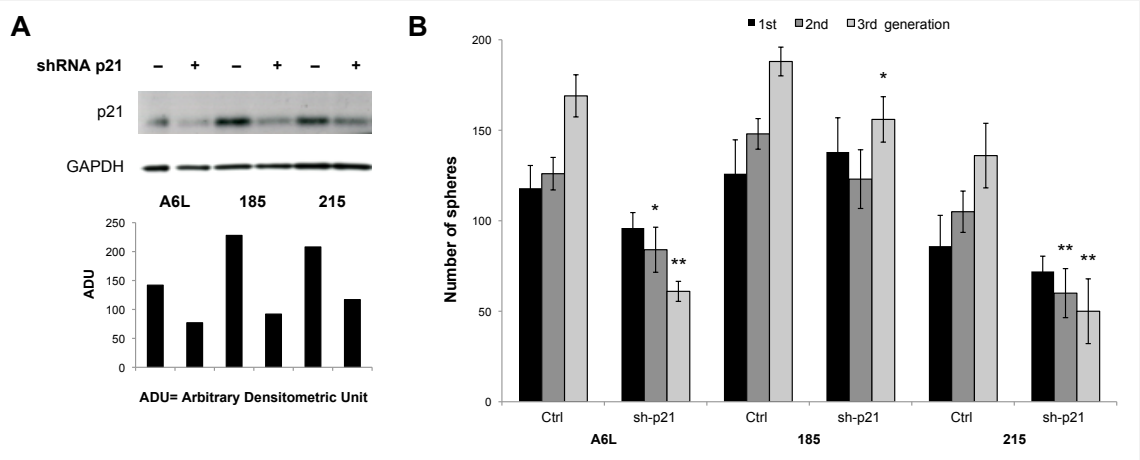



**Figure 32. (A)** Western blot analysis of p21 in cells infected with lentivirus shp21 compared with scramble (**upper panel**) and densitometric quantification of protein bands (**lower panel**). (**B**) Number of spheres during three passages of shp21 cells compared with scrambled cells.

Cell cycle analysis of shp21 cells show increased in the overall proliferation of the cells, as determined by cell cycle analysis (**Figure 33A**). Moreover, p21 knockdown resulted in a more chemosensitive phenotype after treatment with Gemcitabine (**Figure 33B**). Therefore, we were able to recapitulate some of the phenotypes observed in the miR-19-72 over-expression studies by merely silencing p21 as one of the many targets of the miR-19-72 cluster.

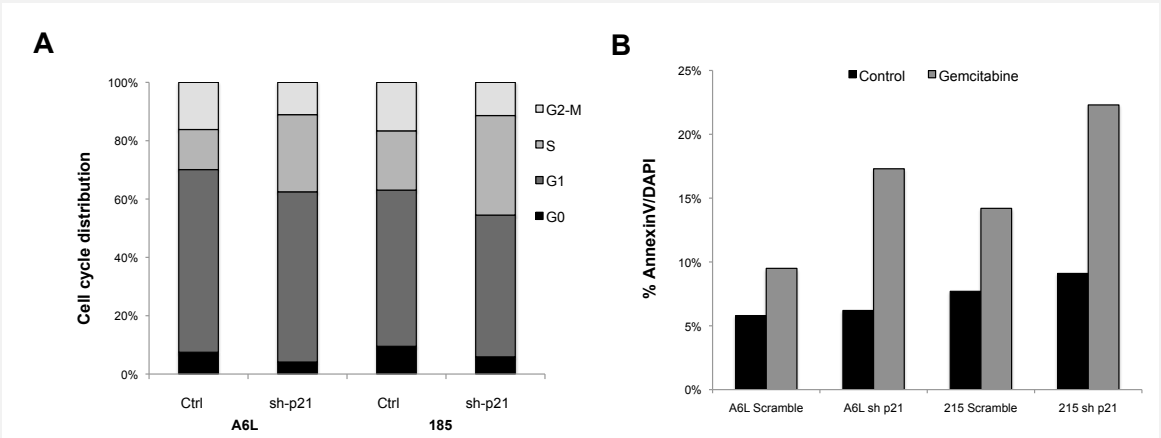



**Figure 33. (A)** Cell cycle analysis using Ki67 by flow cytometry. (**B**) Chemoresistance by Annexin V/Dapi staining after treatment with Gemcitabine in shp21 cells compared with scrambled cells.

Therefore, p21 appears to have a dominant role in CSCs and targeting p21 may have important anti-CSCs consequences. Thus, to validate our findings *in vivo*, we injected limiting dilutions of sh-scramble and sh-p21 cells into immunocompromised mice to assess the tumourigenic capacity of pancreatic CSCs lacking p21. Intriguingly, *in vivo* tumourigenicity was significantly impaired, particularly when low numbers of cells were injected (**Figure 34**).

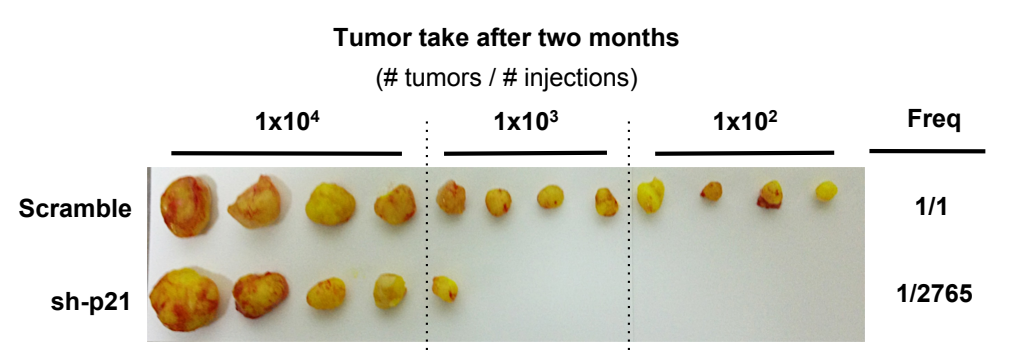



**Figure 34.** In vivo tumorigenicity with limiting dilution of sh-p21 cells compared with scramble cells

This result is consistent with the effect of miR-17-92 over-expression, which further supports that the suppression of p21 might be the key player by which the miR-17-92 cluster regulates self-renewal and chemoresistance of pancreatic CSCs together with the abrogation of the Nodal/ TGF-β1 pathways.

## 7.2. Tbx3 regulates self-renewal capacity and metastasis

TBX3 is a transcription factor that belongs to the T-box gene family and contain a conserved DNA-binding domain called the T-box (Bamshad et al., 1997). TBX3 plays an essential role in embryogenesis and stem cells (Pirity and Dinnyes, 2010) and very recently has been also found overexpressed in several cancers, including breast, pancreatic, liver, bladder, and melanoma, and there is strong evidence linking it to the oncogenic process (Renard et al., 2007, Fan et al., 2004, Peres and Prince, 2013). In order to evaluate if TBX3 is important for pancreatic cancer stem cells, we analyzed spheres derived cells and we observed that TBX3 is

upregulated both at protein (**Figure 35A**) and at RNA level (**Figure 35B**) compared with adherent cells.

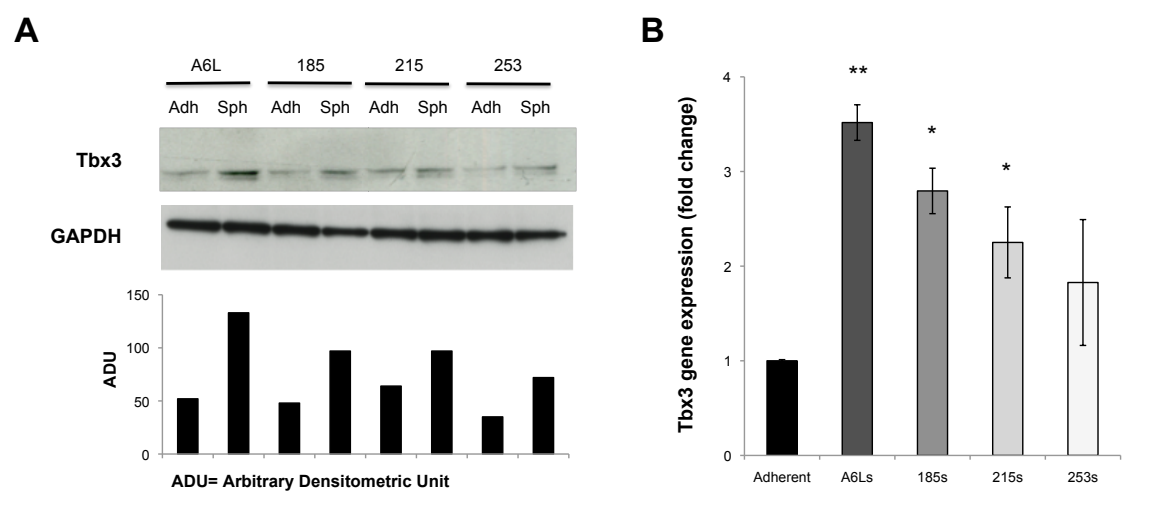



**Figure 35**. **(A)** Western blot analysis of TBX3 comparing adherent and spheres from several primary pancreatic cancer cells (**upper panel**) and densitometric quantification of the western blot (**lower panel**). **(B)** QPCR analysis for TBX3 in spheres comparing with adherent cells.

Moreover, we corroborated our results in a different set of CSC comparing the expression of TBX3 in CD133 sorted cells (**Figure 36A**) and in ALK4 sorted cells (**Figure 36B**). TBX3 resulted upregulated in both CSC enriched populations.

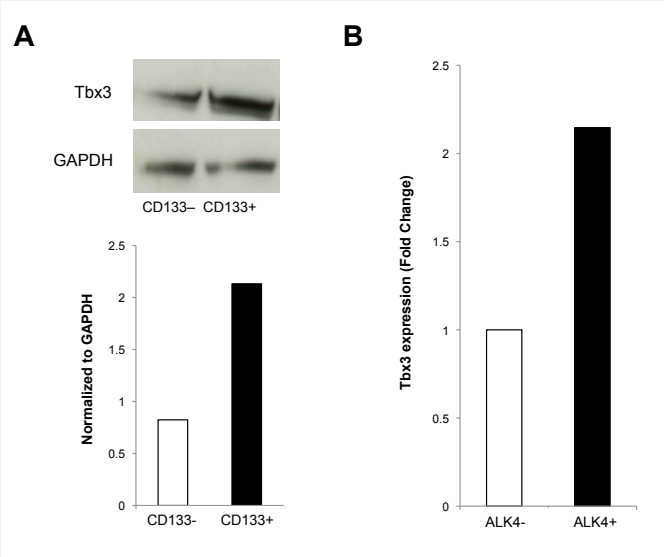



**Figure 36**. **(A)** Western blot analysis of TBX3 in CD133 positive and negative cells (**upper panel**) and densitometric quantification of the western blot (**lower panel**). **(B)** QPCR analysis for TBX3 in ALK4 sorted cells.

Next we asked whether specific knockdown of TBX3 could affect the cancer stem cells population, inhibiting the mechanisms of self-renewal, metastasis and tumourigenicity. Using a lentiviral vector expressing two different shRNA against TBX3, we obtained stable spheres derived cells with TBX3 knockdown (**Figure 37A**). Flow cytometry analysis of sh-TBX3 cells, shows a significant reduction of CSC surface markers CD133 and CXCR4 compared with scramble cells (**Figure 37B**).

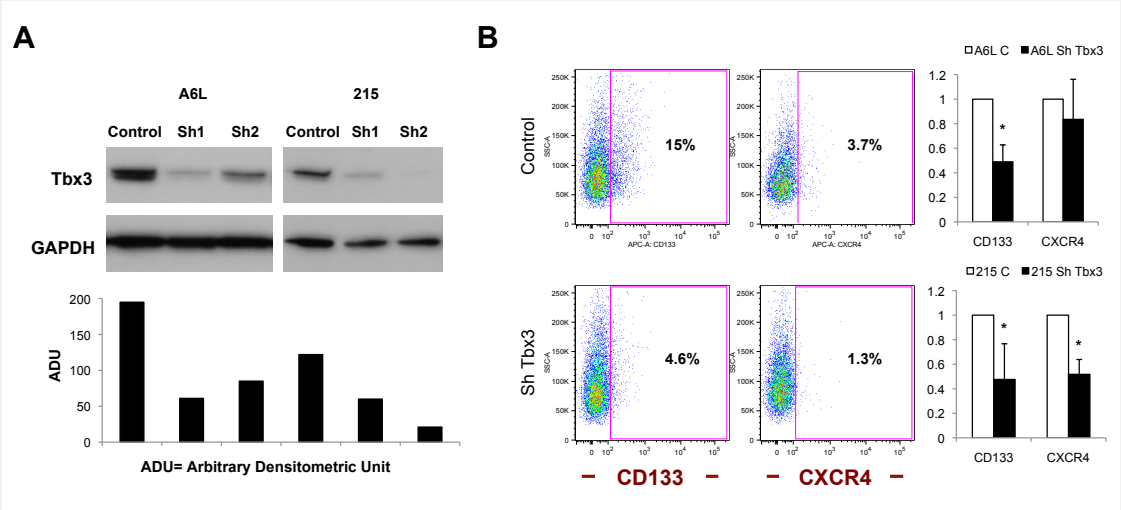



**Figure 37**. **(A)** Western blot analysis of TBX3 in cells infected with lentivirus expressing sh1 and sh2 (**upper panel**) and densitometric quantification of the western blot (**lower panel**). **(B)** Flow cytometry analysis for CSC surface markers CD133 and CXCR4 in cells expressing sh-TBX3 compared with scramble.

Very recently TBX3 has been identified as TGF-β1 target with a double function: inhibition of proliferation and promotion of the migration of breast epithelial cells (Li et al., 2013). Based on these findings, we decided to test migration and invasion capacity of knockdown cells after stimulation with TGF-β1, Nodal and Activin.

## 8. TARGETING QUIESCENT CSCS AS A NOVEL THERAPEUTIC APPROACH FOR PANCREATIC CANCER

Since the sum of our data show that the miR-17-92 cluster possesses strong inhibitory effects on CSC phenotypes by targeting key factors essential for the stem-like nature of CSCs, we finally aimed to test the translational relevance of our findings. Thus, we performed *in vivo* therapeutic intervention studies by inducing over-expression of miR-17-92 in established pancreatic tumors using a doxycycline-switchable system. First, we implanted transduced, but doxycycline naïve cells into immunocompromised mice and once tumors had formed (~100mm$^3$), doxycycline was administered to induce miR-17-92 expression. In addition, some mice were also treated with Gemcitabine (biweekly 125 mg/kg i.p.) from day 14 to 64, to mimic standard of care. Intriguingly, while no significant differences were observed between Lenti-control tumors and Lenti-miR tumors with respect to bulk tumor growth when the 17-92 cluster was expressed post tumor formation, tumors over-expressing the miR-17-92 cluster were significantly more sensitive to Gemcitabine compared to control tumors (**Figure 38A**). In addition, analysis of tumor weight showed a significant reduction for tumors over-expressing miR-17-92 and additionally treated with gemcitabine (**Figure 38B**).

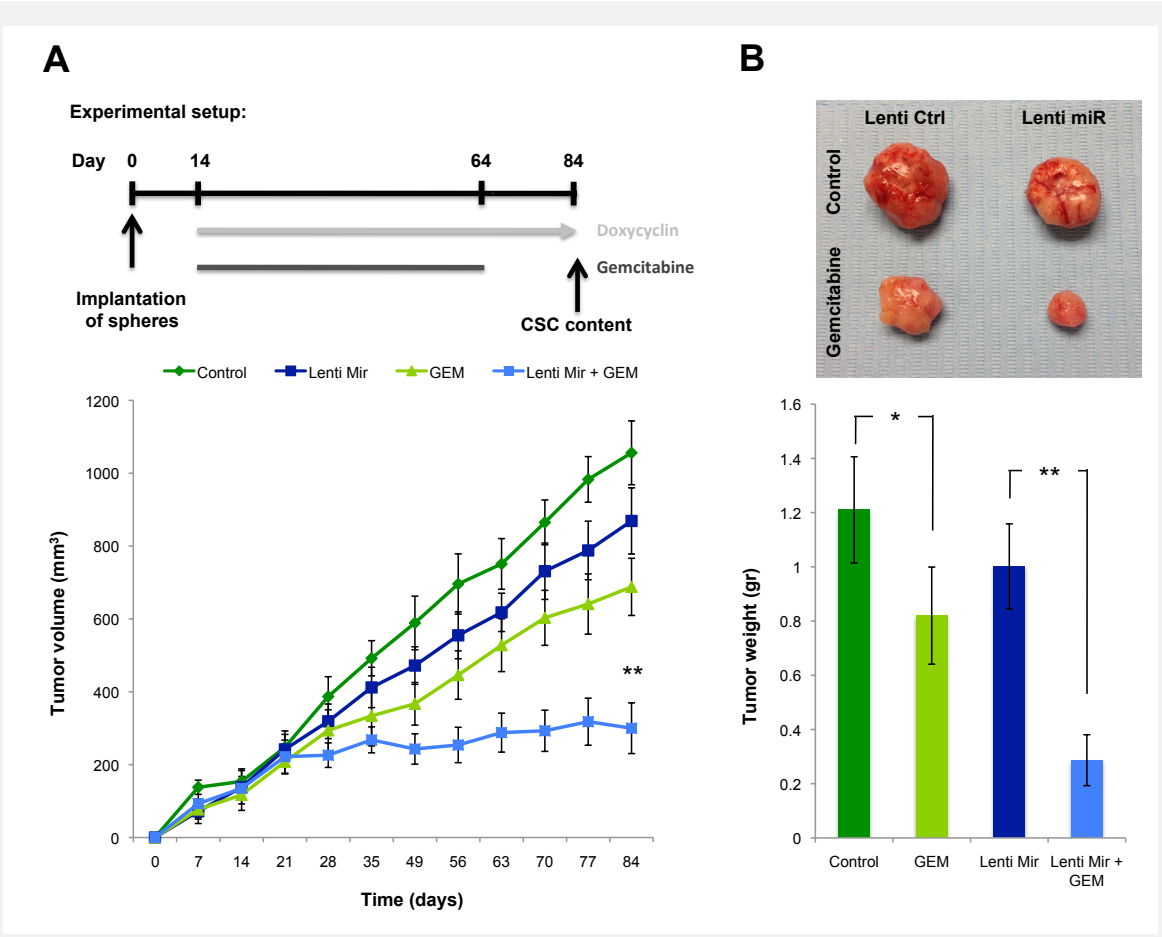
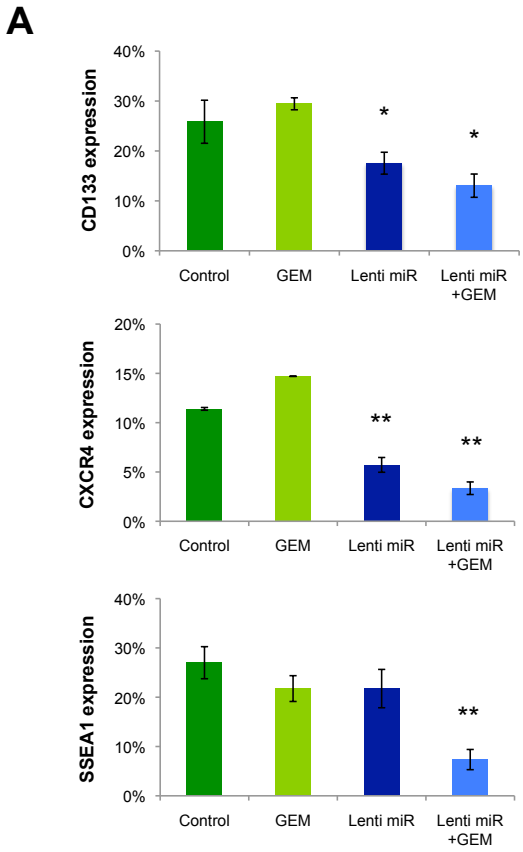



**Figure 38. (A)** Experimental setup for in vivo treatment **(upper panel)** and treatment effects of gemcitabine in miR-17-92 overexpressing cells in established tumors derived from 185 cells in immunocompromised mice **(lower panel).** The mean tumor volume is given; n 6 tumors per group. P value was determined by Student t test. (**B**) Representative picture **(upper panel)** and quantification of tumor weight **(lower panel)**

Importantly, flow cytometry analysis after digestion of the tumours, shows a significant decrease in CSC markers CD133, CXCR4 and SSEA1 in tumours overexpressing miR-17-92 and treated with gemcitabine (**Figure 39A**). Moreover, injection of isolated cells from the tumours in a serial dilution manner further enhanced this difference, showing reducing tumourigenicity of cells overexpressing miR-17-92 and treated with gemcitabine meaning that high levels of miR17-92 forced the cells in a more differentiated and proliferative state that sensitized them to chemotherapy, reducing their self-renewal capacity *in vivo* and at the same time sensitizing them to chemotherapy (**Figure 39B**).




B

**Tumor take after two months**
(# tumors / # injections)

| | $10^4$ | $10^3$ | $10^2$ | Freq |
|---|---|---|---|---|
| Ctrl | 5/5 | 4/5 | 3/5 | 1/360 |
| GEM | 5/5 | 4/5 | 4/5 | 1/289 |
| Lenti miR | 4/5 | 2/5 | 2/5 | 1/3150 |
| Lenti miR + GEM | 2/5 | 2/5 | 0/5 | 1/10739 |

**Figure 39.** (**A**) Flow cytometry analysis for CSC markers CD133, CXCR4 and SSEA1 in cells isolated from the tumors (**B**) In vivo tumorigenicity of cells isolated from the tumors in a serial dilution assay.

# DISCUSSION

Patients with pancreatic ductal adenocarcinoma are still suffering from a devastating prognosis due to lack of early symptoms, reliable methods for detection and early metastatic spread. Current available treatments are not very effective due to intrinsic chemoresistance and only small advances have been achieved in terms of new therapeutic approaches, while still eventually most of the patients succumb from disease resulting in a dismal 5-year survival rate of below 5%. Recent studies on pancreatic cancer identified a subpopulation named cancer stem cells (CSCs), which possess exclusive tumourigenicity based on their unlimited self-renew capacity and recapitulate the cellular heterogeneity of the parental tumour. Of Clinical utmost importance is their strong resistance to conventional chemotherapeutics compared to their differentiated progenies resulting in tumour recurrence following initial tumour regression in response to anticancer chemotherapy. Therefore, elimination of the more differentiated cancer cells, even if associated with significant tumour size reduction, is unlikely to lead to the eradication of the tumourigenic potential of the tumour, as this is restricted to the CSC population. These findings have opened a new research field focussing on a better understanding of CSC biology and have spurred renewed hope for the development of novel and more effective CSC-specific targeted therapies in combination with effective treatments against bulk tumour cells. To achieve the latter, it is essential to elucidate the signalling and regulatory mechanisms that are unique to CSCs.

Previous studies, including our own have shown that pancreatic cancers contain a rare population of undifferentiated cells that are characterized by expression of CD133 or CD44/CD24 (Li et al., 2007b, Hermann et al., 2007b). Moreover, it has also been shown, that it is possible to enrich and expand pancreatic CSCs in vitro as anchorage-independent spherical colonies termed spheres. These spheres are composed of a subset of cells with stem cell-like properties including the ability to form secondary spheres as well as more differentiated progenies. Molecular and biological characterization of pancreatic CSCs using these in vitro as well in vivo approaches indicates that several pathways are indispensable for the maintenance of their self-renewal capacity and subsequently also for their exclusive in vivo tumourigenicity. Specifically, the Nodal/Activin pathway is essential for the regulation of stemness properties of pancreatic CSCs. The ligands are expressed in pancreatic CSCs regulating self-renewal and metastasis, but also in activated pancreatic stellate cells (PSCs), which are abundantly present in the stroma surrounding pancreatic cancer cells and serve as a CSC niche (Lonardo et al., 2011, Lonardo et al., 2012). Building on these original findings,

in the first part of this Doctoral Thesis, we aimed to better characterize pancreatic CSCs using different approaches in order to discover new regulatory pathways with particular emphasis on self-renewal and chemoresistance, respectively.

For this purpose, we first analysed sphere-derived cells enriched for cancer stem cells and found that besides the over expression of surface CSCs markers (CD133, CXCR4 and SSEA1) and pluripotency associated genes (Oct4, Sox2, Klf4 and Nanog), they also possess a specific cell cycle signature with an increase both at RNA and protein level of p21, p27 and p57 that are involved in the maintenance of a quiescent or slow proliferating phenotype. Further experiments using cell cycle analysis and the label-retaining technique, which is based on the retention of the lipophilic dye PKH26 conclusively demonstrated the presence of a slow proliferating population bearing all the characteristics of CSCs.

Secondly, we focussed our attention on chemoresistant CSCs both *in vivo* and *in vitro*: we found that gemcitabine leads to a relative increase in the number of CSCs as evidenced by increased expression of CSC surface markers and enrichment of a cancer stem cell-like gene signature, indicating a preferential targeting of more differentiated cells. Moreover, cell cycle analysis showed that rapidly proliferating cancer cells were quite efficiently eliminated by treatment with gemcitabine, while more quiescent cells survived; an observation that is in line with previous findings (Scopelliti et al., 2009).

Self-renewal and chemoresistance are both highly orchestrated processes that require complex transcription and posttranscriptional regulation of gene expression. miRNAs target multiple genes and play important roles in many cancer processes. In an effort to discover targetable signalling pathways present in drug-resistant CSCs, we next examined miRNA expression signatures enriched by chemotherapy in primary pancreatic cancers cells. RNA extracted from sphere-derived cells and gemcitabine-resistant tumours and cells, respectively showed a specific and common miRNA expression profile across several tumours tested.

Specifically, we found that the miR-17-92 cluster, composed by six members miR-17, -18a, 19a, 19b, 20a and -92a, is markedly suppressed in sphere-derived cells and chemoresistant pancreatic CSCs as compared to their more differentiated counterparts. These findings are intriguing as the cluster is regularly overexpressed in cancer (reference) and the oncogenic nature of miR-17-92 activation is well established (He et al., 2005, O'Donnell et al., 2005), Loss of heterozygosity at 13q12-q13 is associated with multiple tumour progression and poor prognosis, including breast cancer, squamous cell carcinoma of the larynx, retinoblastoma,

hepatocellular carcinoma and nasopharyngeal carcinoma (Xiang and Wu, 2010, Coller et al., 2007) and deletion of *miR-17-92* cluster was observed in a relevant percentage of ovarian cancers, breast cancers and melanomas (Zhang et al., 2006). However, the *miR-17-92* cluster has also been demonstrated to act as a tumour suppressor in breast cancer cells (Hossain et al., 2006), in gastrointestinal stromal tumours (Gits et al., 2013) and in oral squamous carcinoma (Chang et al., 2013). These observations may reflect the functional complexity of mir-17-92 rendering the biological response dependent on tumour type and cell type.

Even more importantly, the underlying targets and signalling cascades that are deregulated in response to modulation of miR-17-92 still remain largely elusive as of today. Strikingly, studies that mostly aimed at determining miR-17-92 targets have focused on individual members of the cluster, despite the observation that the entire cluster is regularly activated in cancer (references). Specifically, different members?? of the cluster have recently been shown to target diverse pathways, including TGF-β1 (Dews et al., 2010) and HIF-1α signalling (Taguchi et al., 2008), but their role in the regulation of pancreatic cancer and pancreatic cancer stem cells in particular remained to be determined.

Our data now demonstrate in a large set of primary cells and (fresh) primary patient tissues, that inhibition of miR-17-92 by means of specific antagomirs in more differentiated cells equipped them with CSCs features that are regularly restricted to bonafide CSCs. This was evidenced by up-regulation of CD133, increased sphere formation capacity, reduced proliferation with subsequent increase in chemoresistance. Most importantly, antagomirs 17-92 significantly promoted their *in vivo* tumourigenicity and in vivo resistance to chemotherapy.

On the contrary, in gain-of-function experiments using a lentiviral approach, we overexpressed miR-17-92 in sphere-derived cells in order to push the cells in a more differentiate state. Indeed, we observed a significant decrease in sphere formation capacity, a reduction of CSCs surface marker expression and increased proliferation. The latter finding was most intriguing as it resulted in subsequent exhaustion of the slow cycling cells, wich was reflected in increased sensitivity for treatment with gemcitabine. Moreover, it has previously been demonstrated that acquisition of an invasive phenotype represents a critical initiation step in the process of pancreatic cancer cell dissemination (Rhim et al., 2012) and that a subset of CSCs possess particular metastatic capabilities (Hermann et al., 2007b). Therefore, we next tested migration and invasive capacity of sphere-derived cells in the presence or

absence of miR-17-92 following stimulation with Nodal, Activin and TGF-β1, which we have demonstrated as main drivers of the metastatic phenotype in pancreatic cancer (Lonardo et al., 2011). Overexpression of miR-17-92 family members significantly reduced migration and invasion capacity *in vitro*, and more importantly we also demonstrate the complete inhibition of liver metastasis *in vivo* after intrasplenic injection of sphere-derived cells overexpressing miR-17-92. Subsequent in vivo experiments showed a reduction of tumourigenicity in dose dependent manner and exhaustion of the CSCs pool during serial in vivo passaging, which most likely is related to the extensive proliferation of the cells overexpressing miR-17-92 and subsequent accumulation of DNA damage due to alteration of mechanism of DNA damage repair (see below).

While the aforementioned studies establish the miR-17-92 cluster as a key negative regulator of pancreatic CSCs function. Next step was to identify specific targets that could be linked to the strong phenotype observed in response to modulation of miR-17-92 cluster expression. For this purpose, we used Targetscan software to define putative single miRNA targets. Intriguingly, we were able to identify several targets clustering around pathways that previously have been associated with the regulation of CSCs; e.g. Nodal/Activin pathway and cell cycle regulators. Specifically, we found miR-18a to regulate both SMAD2 and SMAD4, two key components of the Nodal/Activin signalling cascade, suggesting that miR-18a substantially contributes to deregulation of this CSC pathway by regulating a selected set of target genes. Moreover, miR-17 and miR-20a showed a more wide range of targets since they are able to inhibits TGFBR2, ALK4, SMAD4, as well p21 and TBX3, all of which are again linked to Nodal/Activin signalling and cell cycle regulation, respectively. Finally, miR-92a was shown to also target TBX3, but also p57. Therefore, our data demonstrate that miR-17-92 dampens Nodal/Activin signalling in a multifaceted way by acting both upstream and downstream of pSMAD2/SMAD4, further underscoring its ability to regulate multiple components of the same CSC pathway. The ability to simultaneously target different components of the signalling cascade, as well as the downstream effectors through multiple miRNAs belonging to the same cluster, allows for a very tight control of the Nodal/Activin transcriptional programme. Moreover, it renders the cells with strong plasticity for the regulation of different Nodal/Activin target genes (**Figure 40**)

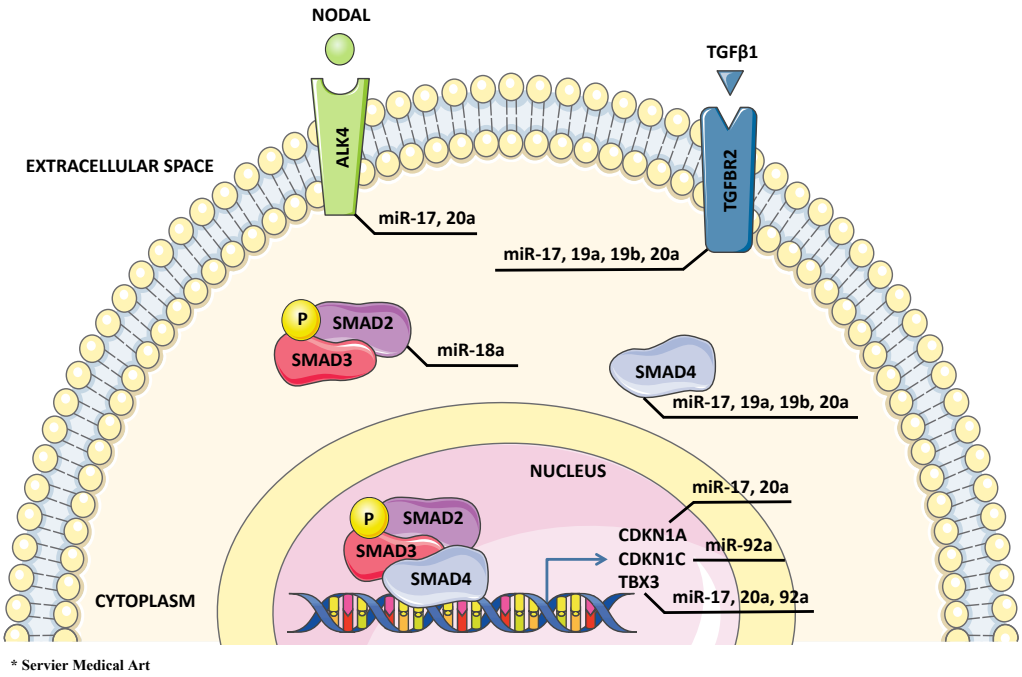



**Figure 40**. Graphical illustration of miR-17-92 targets in pancreatic cancer stem cells

We observed that p21 was highly expressed in sphere-derived cells as well as chemoresistant cells compared to their respective counterparts. This result suggests that in the context of CSC biology, p21 may serve to maintain tumour-initiating potential by keeping their proliferative activity in check. Indeed, there is strong evidence from murine models of normal hematopoietic and leukemic stem cells that p21 is an important regulator of self-renewal. In the absence of p21, both normal hematopoietic stem cellas as well as transformed leukemic stem cells underwent functional exhaustion and were unable to maintain the clone (Cheng et al., 2000, Viale et al., 2009). Our data now also support a role for p21 in the prevention of pancreatic CSCs exhaustion through cell-cycle restriction, which resulted in the accumulation of DNA damage as evidenced by increased gH2AX foci and may result in the elimination/dysfunction of the cells via mitotic chaos. These results are consistent with a number of publications that have recognized a role for p21 in the protection of cancer cells from stress and DNA damage (Gorospe et al., 1996, Tian et al., 2000, Bene and Chambers, 2009). Importantly, we validated these results using an shRNA approach and observed that

p21 knockdown consistently affected the self-renewal capacity as well chemoresistance of pancreatic CSCs. Finally, we also show that the Nodal/Activin/TGFb-mediated increase in p21 expression is Smad2 dependent manner.

Considering the widespread expression of Alk4 in CSCs and the functional involvement of Alk4 in mediating CSC self-renewal and invasiveness, the suppression of Alk4 by miR-17-92 reveals a previously unknown epigenetic regulation in pancreatic CSCs. Even more important from a clinical point of view, abrogation of Nodal/Activin signalling via miR-17-92 overexpression could be a promising and highly specific therapeutic approach, as it would not only result in reduced expression of Alk4, but also induce direct miR-17-92-mediated repression of Nodal/Activin responsive genes. The latter would avoid the bias of the SMAD4 status as Smad4 is mutated in about 50% of pancreatic tumours (Schneider and Schmid, 2003) and therefore a significant proportion of tumours may not be responding to pharmacological inhibition of Alk4/Alk7 by specific inhibitors.

# CONCLUSIONS

We provide compelling functional evidence for downregulation of miR-17-92 in preferentially quiescence and chemoresistance in pancreatic CSCs. In summary we found:

1. Spheres-derived cells are enriched for CSCs as evidenced by enhanced expression of surface markers CD133, CXCR4 and SSEA1, pluripotency-associated genes (Oct4, Sox2, Klf4, Nanog), a slow cycling phenotype with increased expression of p21, p27 and p57 and increased chemoresistance;
2. Treatment with Gemcitabine enriched for CSCs with above stem-like gene signature and slow proliferation profile both *in vivo* and *in vitro;*
3. Sphere-derived cells and chemoresitant cells show a consistent and specific miRNA expression profile with a significant downregulation of miR-17-92 family;
4. Inhibition of miR-17-92 by antagomirs in differentiated cancer cells induced a CSCs phenotype accompanied by increased expression of CD133, enhanced sphere formation capacity, reduced proliferation and increased chemoresistance;
5. Lentiviral overexpression of miR-17-92 in CSCs reverted the CSCs phenotype as evidenced by reduced surface expression of CD133 and CXCR4, deminished sphere formation capacity, impaired invasiveness in vivo and in vitro, reduced tumourigenicity and eventually exhaustion during serial in vivo passaging;
6. We identified several target genes that belong to the Nodal/Activin/TGFb signalling pathways (ALK4, SMAD4, SMAD2) as well as regulators of the cell cycle (p21 and p57) and self-renewal and metastasis (TBX3);
7. shRNA-mediated knock down of p21 diminished self-renewal capacity, cell cycle progression and chemoresistance as well in vivo tumorigenicity;
8. shRNA-mediated knock down of TBX3 affect CSCs self-renewal and invasive capacity.

# CONCLUSIONES

Con esta tesis demostramos grandes evidencias funcionales donde se observa que la expresión de miR-17-92 está inhibida de manera más frecuente en células quiescentes y quimioresistentes de las CSCs pancreáticas. En resumen, hemos demostrado:

1. Las células obtenidas de esferas están enriquecidas en CSCs, ya que la expresión de los marcadores CD133, CXCR4 y SSEA1 es mayor, como también los genes asociados a pluripotencia (Oct4, Sox2, Klf4, Nanog).Además también se evidencia un fenotipo con ciclo celular lento, con aumento de la expresión de p21 y p57, asi como también un aumento de la quimioresistencia;
2. El tratamiento con la Gemcitabina enriquece en CSCs debido a que aumentan la expresión de los genes asociados a pluripotencia y decrece el patrón de proliferación tanto *in vivo* como *in vitro;*
3. Las células derivadas de esferas y las células quimioresistentes muestran un perfil de expresión de miRNA específico y consistente con una inhibición de la expresión de la familia de miR-17-92;
4. La inhibición de miR-17-92 con antagomirs en células cancerígenas diferenciadas induce un fenotipo asociado a célula troncal cancerígina acompañado con aumento de la expresión del marcador CD133, mayor capacidad de formar esferas, reducción de la proliferación y aumento de quimioresistencia;
5. La sobreexpressión de miR-17-92 mediante un lentivirus en las CSCs reverte el fenotipo de CSC debido a que se evidencia una reducción de la expresión de los marcadores CD133 y CXCR4, baja la capacidad de formación de esferas, disminuye la capacidad de invasion in vivo e in vitro, se reduce la tumorigenicidad y eventualmente ocurre un reducción de formación de tumores en pases seriados a lo largo del tiempo;
6. Hemos identificado distintos genes que pertenecen a las vías de señalización de Nodal/Activin/TGFb (ALK4, SMAD4, SMAD2), asi como reguladores del ciclo celular (p21 y p57) y también los implicados en la capacidad de auto-replicación y metástasis (TBX3);
7. Los shRNA que median la inhibición de p21 bajan la capacidad de auto-replicación, la progresión celular y la quimioresistencia, asi como la tumorigénesis in vivo;
8. Los shRNA que median la inhibición de TBX3 afectan la capacidad invasiae y de auto-replicación de las CSCs.

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